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ACUTE HAEMORRHAGIC LEUCO-ENCEPHALITIS

BY

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(RECEIVED FOR PUBLICATION AUGUST 25, 1953)

It was in 1940 that Weston Hurst (1941) encountered two cases of fatal encephalitis which he regarded on clinical and pathological grounds as representing a previously unidentified entity to which he gave the name of acute haemorrhagic leuco-encephalitis. To quote Hurst's original description, the disease was "an acute cerebral condition, virtually localized as far as the cerebrum was concerned to the white matter, and developing more or less abruptly in apparently normal individuals. Perivascular necroses, perivascular and focal demyelination, haemorrhages, oedema, and cellular infiltration were the chief components of the pathological picture."

In the 12 years since Hurst's paper appeared remarkably few similar cases have been reported, and in fact only five further examples have been described under the same name. These have been recorded by Henson and Russell (1942), Shallard and Latham (1945), MacArdle, van Bogaert, and Lhermitte (1949, two cases) and by Greenfield (1950). It so happens that three characteristic examples of the disease have occurred in the neurosurgical department of St. George's Hospital over a two-year period, and it seems probable that the condition is not such an extreme rarity as the small number of recorded cases would indicate. The object of the present communication is to put on record these three further cases (bringing the number recorded up to 10) and to draw the attention of general pathologists to the existence of this variety of encephalitis. The descriptions of the disease which follow are based on the three new cases together with the seven previously reported. More detailed summaries of the three cases are included in an appendix to this paper.

Clinical Features

The salient clinical features are summarized in Table I. The age of the patients has varied from 2 years 10 months (Case 2 of the present series) to 51 years (Greenfield, 1950), but seven of the 10 patients were young adults in the third and fourth decades. The sex incidence shows a male preponderance with seven male and three female patients. The duration of the disease has proved variable. In seven cases the onset of neurological signs was preceded by a prodromal period of upper respiratory infection lasting from as little as two days up to about 15 days, and in four cases the prodromal infection was separated from the encephalitic stage by a few days of apparent recovery. The longest survival after the onset of

TABLE I
SUMMARY OF CLINICAL COURSE

Author	Age (yrs.)	Sex		Duration (Days)		
				Prodromal Stage	Interval	Neurological Stage
Hurst (1941): Case 1 ..	33	F	Right flaccid paralysis, speech loss, coma	0	0	2
„ 2 ..	33	M	Sore throat, fever, speech loss, fit, coma	10	11	6
Henson and Russell (1942)	21	M	Cold, right hemiparesis, decerebrate rigidity	3	8	5
Shallard and Latham (1945)	7	F	Cold, right hemiparesis, coma, flaccidity	2	0	4
MacArdle *et al.* (1949): Case 1 ..	22	M	Fits, coma, right hemiplegia	0	0	4
„ 2 ..	37	F	Confusion, speech loss, right-sided fits, coma	0	0	5
Greenfield (1950)	51	M	Bronchitis, drowsiness, right-sided fits, coma	?15	0	4
Present author: Case 1 ..	28	M	Cold, vomiting, coma, ventricular shift to right	4	8	4
„ 2 ..	2 10/12		Laryngitis, vomiting, fits, left hemiplegia, coma	3	0	5
„ 3 ..	31	M	Bronchitis, drowsiness, coma	2	3	5

neurological signs was six days, while Hurst's original patient died after only 42 hours' illness.

A detailed account of the symptoms and signs is beyond the scope of this paper. Striking features, however, are the early onset of coma and the frequent gross asymmetry of the signs. This asymmetry has been conspicuous in nine of the 10 cases and has sometimes led to needling of the brain in search of an abscess or other focal lesion. Six cases have shown right hemiparesis with early speech involvement.

Clinical Pathology

It is unfortunate that few laboratory tests have been recorded on material obtained from these patients during life—a fact no doubt associated with the rapid downhill course of most cases. Such information as is available is summarized in Table II. In the cerebrospinal fluid the cell count varied from normal up to a count of 341 per c.mm. and the elevated counts were always associated with considerable numbers of polymorphonuclear leucocytes, the proportion of these cells always exceeding 70% with total counts over 100 per c.mm. The cerebrospinal fluid protein level was also moderately elevated in most cases, the highest level noted being 140 mg. per 100 ml. (present series, Case 3). The cell and protein increases were approximately proportionate except for the case of Shallard and Latham (1945) in which the cerebrospinal fluid showed a cell count of 200 per c.mm. (90% polymorphs) but a protein level of only 30 mg. per 100 ml. Three observations of the cerebrospinal fluid sugar and chloride levels fall within normal limits. Blood counts were recorded in only three cases, two showing a high and the third a moderate leucocytosis, while two differential counts both revealed polymorphonuclear increase. Examination of the urine was not recorded in five of the cases. Of the others, one showed heavy albuminuria with casts (Henson and Russell, 1942) and one slight albuminuria (MacArdle *et al.*, 1949, Case 2). In the three cases of the present series protein was absent from the urine at a single examination in each.

TABLE II
CLINICO-PATHOLOGICAL FINDINGS

Author	Cerebrospinal Fluid			Blood		Urine
	Cells (per c.mm.)	Polymorphs (%)	Protein (mg./100 ml.)	White Cells (per c.mm.)	Polymorphs (%)	
Hurst (1941):						
Case 1 ..	—	—	—	—	—	—
„ 2 ..	—	—	—	22,900	—	—
Henson and Russell (1942)	0	—	40	12,000	82	Albumin +++
	6	33	35	—	—	Casts ++
Shallard and Latham (1945)	200	90	30	—	—	—
MacArdle *et al.* (1949):						
Case 1 ..	—	—	—	—	—	—
„ 2 ..	6	—	55	—	—	Albumin +
Greenfield (1950)	342	84	60	—	—	—
Present author:						
Case 1 ..	220	84	80	—	—	No protein
„ 2 ..	2	—	30	30,000	81	„ „
„ 3 ..	150	71	140	—	—	„ „

The Naked-eye Lesions

The meninges have usually shown no naked-eye change, but sometimes they appear congested (Henson and Russell, 1942; present series Cases 1 and 3). Greenfield (1950) noted some creamy exudate over the convexity of the left frontal lobe. The most characteristic lesion is seen in the white matter of the cerebral hemispheres and takes the form of haemorrhages varying in size from minute points up to areas a few millimetres in diameter, which, in the more severely involved regions, may become confluent (Fig. 1). These more affected parts show asymmetrical oedematous swelling with

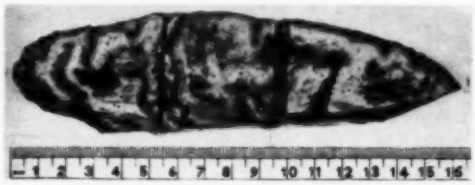

FIG. 1.—Three pieces of the left cerebral hemisphere from Case 1. Petechial haemorrhages, in places confluent, are scattered throughout the white matter and there is haemorrhage in the track of an exploratory needle.

FIG. 2.—Cerebellum and brain-stem from Case 3. There are numerous petechial haemorrhages in the white matter.

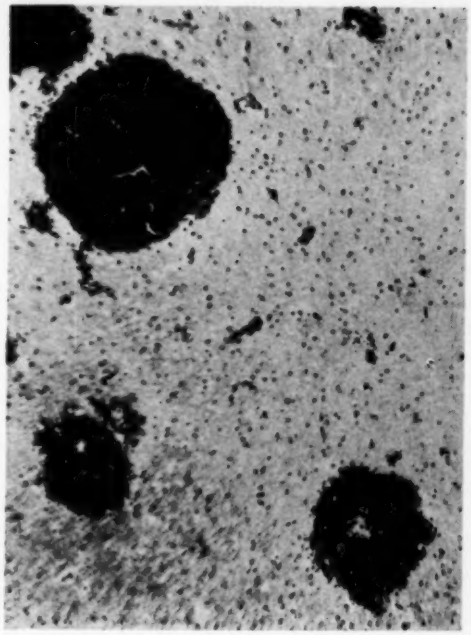

FIG. 3

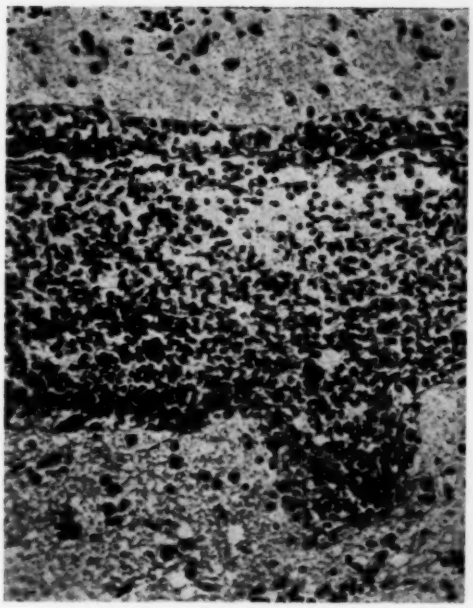

FIG. 5

FIG. 3.—Ball and ring haemorrhages in the parietal white matter (Case 1). Phloxine-tartrazine × 45.

FIG. 4.—Disrupted capillary in the centre of a ball haemorrhage (Case 1). Picro-Mallory × 300.

FIG. 5.—Leucocytic exudate, chiefly polymorphonuclear, in the sheath of a small vein (Case 2). Haematoxylin and eosin × 180.

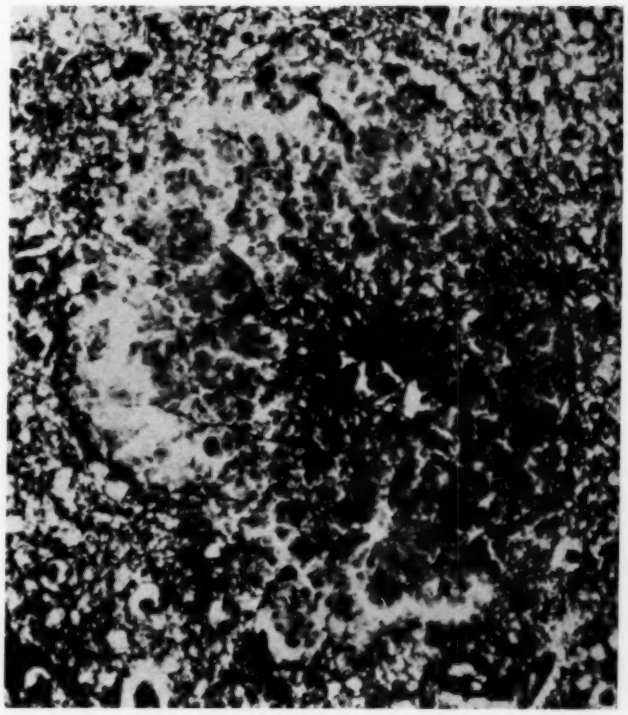

FIG. 4

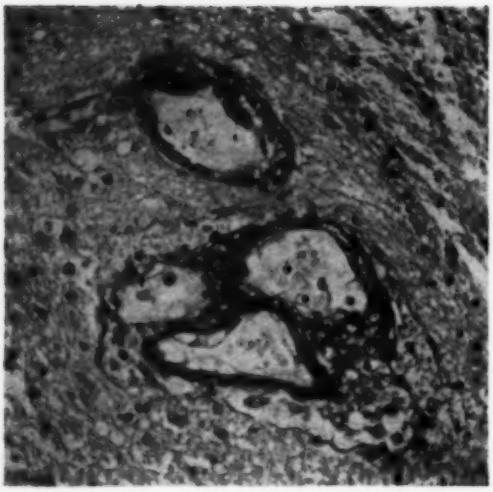

FIG. 6

FIG. 8

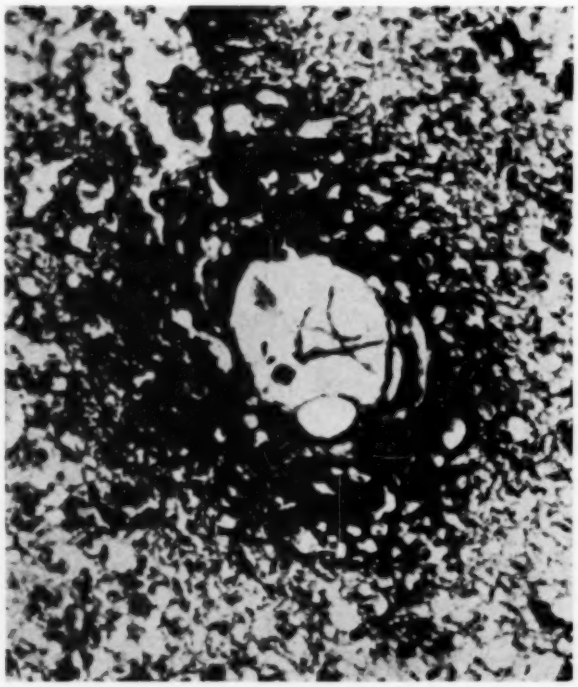

FIG. 7

FIG. 6.—Fibrin impregnation of the walls of venules in the white matter (Case 2). Acid-picro-Mallory × 175.

FIG. 7.—Fibrin impregnation extending through the vessel wall into the adjacent white matter (Case 1). Acid-picro-Mallory × 312.

FIG. 8.—Fibrin deposit around the lining of a venule and extending through the wall to the perivascular sheath (Case 1). Acid-picro-Mallory × 312.

FIG. 9.—Fibrin-plugged capillaries in the white matter (Case 2). Acid-picro-Mallory × 130.

FIG. 10.—Multiple foci of demyelination in the cerebellar white matter (Case 3). Loyez × 35.

FIG. 11.—Demyelination in an area of haemorrhage (Case 3). Loyez × 100.

FIG. 12.—Perivascular demyelination (Case 3). Loyez × 120.

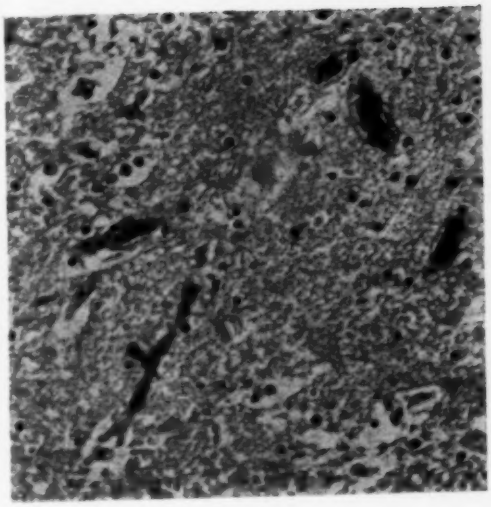

FIG. 9

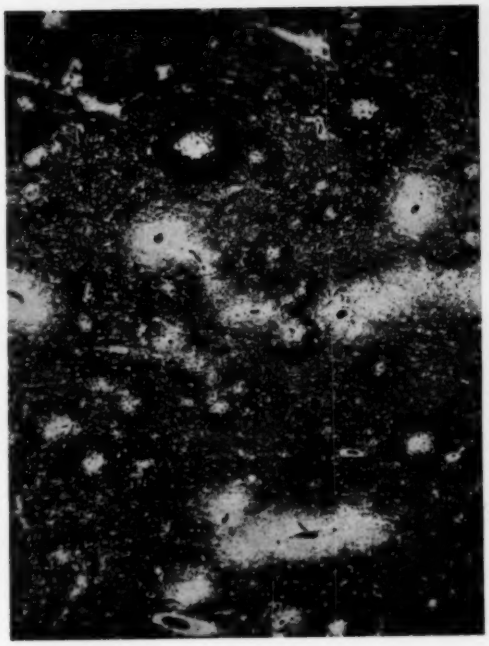

FIG. 10

FIG. 11

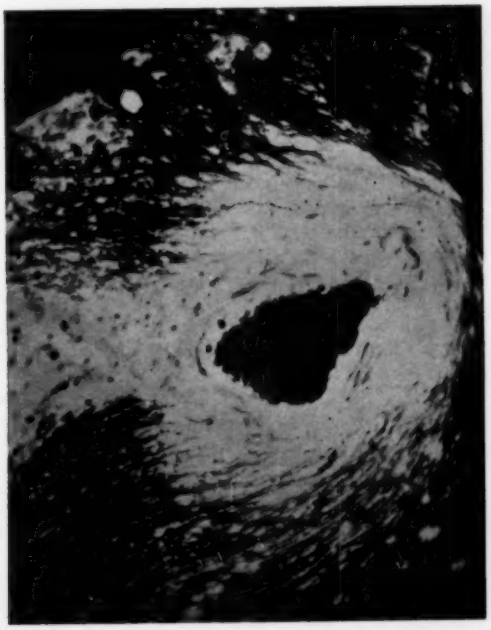

FIG. 12

pinkish or grey discoloration and the most severely hit area may be of an almost gelatinous consistency.

The distribution of these lesions is highly characteristic and is summarized in Table III. The haemorrhages involve the white matter only, the grey matter showing nothing more than some vascular engorgement. The central white matter of one or both cerebral hemispheres is regularly involved and in most cases has shown the most severe lesions. The haemorrhages may extend into the white cores of the convolutions, but the U-fibres between adjacent convolutions are usually

TABLE III
DISTRIBUTION OF NAKED-EYE AND MICROSCOPICAL LESIONS

Author	Areas Affected
Hurst (1941):	
Case 1 ..	Left centrum semi-ovale, corpus callosum, cerebellar peduncles, pons, cerebellum*
„ 2 ..	Left centrum semi-ovale, corpus callosum, right centrum semi-ovale,* mid-brain,* pons*
Henson and Russell (1942)	Central third of white matter of both cerebral hemispheres, corpus callosum,* pons*
Shallard and Latham (1945)	Central white matter of left cerebral hemisphere, cerebellum*
MacArdle *et al.* (1949):	
Case 1 ..	Central white matter of both hemispheres, cerebral peduncles, pons, cerebellum*
„ 2 ..	Central white matter of both hemispheres, left more affected than right
Greenfield (1950)	Central white matter of both hemispheres, corpus callosum, pons*
Present author:	
Case 1 ..	White matter of both hemispheres, mid-brain, pons
„ 2 ..	Left temporal white matter, central white matter of both hemispheres
„ 3 ..	Central white matter of both hemispheres, mid-brain, cerebral peduncles, cerebellum, pons

* These regions showed microscopical lesions only.

spared. The corpus callosum has shown naked-eye lesions in half the cases. The level to which the lesions descend has varied greatly in the 10 cases. They have been noted in the cerebral peduncles in three cases and in the pons in four, but Case 3 of the present series is the first in which they are recorded in the white matter of the cerebellar hemispheres (Fig. 2), though other cases have shown microscopic lesions there.

Histopathology

The distribution of the microscopic lesions (Table III) is more widespread than of the naked-eye lesions, but the former, like the latter, are virtually limited to the white matter. They extend over a wider area of the hemispheres and to a lower level in the brain stem, the cerebellum being quite frequently involved (three cases). It is convenient for descriptive purposes to consider the different types of microscopic lesion separately, but it must be emphasized that a combination of lesions may occur at a single site: for example, haemorrhage, demyelination, and neurofibril disintegration.

Haemorrhage.—The haemorrhages are the most conspicuous lesion in ordinary preparations. Their structure is best studied in less severely affected areas where they are small and discrete. Two types are described : ball haemorrhages and ring haemorrhages (Fig. 3). The ball haemorrhage is a minute sphere of extravasated blood, and serial sectioning will usually reveal a disrupted capillary in its centre (Fig. 4), but the ring haemorrhage has a non-haemorrhagic centre in which it is often possible to find a capillary plugged with fibrin. Owing to the rapidly fatal course of the disease the extravasated red corpuscles appear intact in sections and have not lost their haemoglobin.

Two types of exudate may also be distinguished, cellular exudate and serous exudate. A moderate collection of leucocytes may be seen in the perivascular space around many of the small venules in an affected part (Fig. 5). In transverse section the cells often form an asymmetrical bulge, breaking out from the vessel sheath and spreading into the adjacent brain tissue. A high proportion of the cells are neutrophil polymorphonuclear leucocytes. There is a varying admixture of lymphocytes and histiocytes, but plasma cells and eosinophils have not been noted.

The appearance referred to as " serous exudate " is a rather striking finding seen in all three cases of the present series and well illustrated by Henson and Russell (1942). It consists of homogeneous, indifferently staining material distending the perivascular sheath and in places bursting out into the adjacent white matter. Sometimes there is considerable contamination with red blood corpuscles.

Lesions in Blood Vessels.—The lesions in the blood vessels constitute what are perhaps the most characteristic microscopic features of the disease. The small venules of the white matter are the vessels most affected but there are lesions also in the capillaries and sometimes in small arterioles. The most characteristic lesion is shown in Fig. 6. It consists of impregnation or replacement of the tissues forming the vessel wall with material giving the staining reactions of fibrin, while similar material is present in varying amounts in the perivascular space. A modification of this lesion is depicted in Fig. 7 in which the fibrin impregnation extends beyond the vessel wall and

perivascular space far into the surrounding white matter. Sometimes the fibrin-impregnated tissue forms a halo outside an intact vessel wall and sheath, while another appearance (Fig. 8) is of a plastering of fibrin around the inside of the vessel wall often extending through the thin wall to the perivascular sheath. Endothelium may grow over the deposit to line the new narrowed lumen, and the whole appearance is then reminiscent of the description given by Duguid and Anderson (1952) of hyaline sclerosis in the splenic arterioles.

Capillary disruption and obstruction in association with the haemorrhages have already been mentioned. Groups of fibrin-plugged capillaries may also be found without any apparent relationship to haemorrhages (Fig. 9).

The application of myelin stains to this material reveals the presence of widespread foci of demyelination (Fig. 10). Closer inspection shows two types of demyelinated area—irregular patches in and around the areas of haemorrhage (Fig. 11) and narrow zones around small blood vessels (Fig. 12). Cells are scanty in the demyelinated areas. Appropriate staining methods show disruption of the neurofibrils in relation to many of the foci of demyelination. Microglial proliferation occurs in the affected areas and in the cases of longer standing fat phagocytes collect around the degenerate foci.

Cellular degeneration affecting pyramidal and Purkinje cells, astrocytes, oligodendroglial cells, ependymal cells, and cells of the choroid plexus was noted by Henson and Russell (1942), but has not usually been conspicuous.

Discussion

The addition of these three further cases to the small group of cases already published strengthens Hurst's contention that acute haemorrhagic leuco-encephalitis is a specific disease entity distinct from other forms of encephalitis. The specific pathological features which justify the classification of a case into this type of encephalitis are multiple capillary haemorrhages, focal and perivascular demyelination, and the characteristic fibrin impregnation of the vessel walls, with a predominantly polymorphonuclear exudate all virtually limited in their incidence to the white matter.

Search of the literature shows few cases other than the seven already considered which fulfil these conditions. Adams, Cammermeyer, and Denny-Brown (1949), however, described under the name of "acute necrotising haemorrhagic encephalopathy" four cases which, though differing from the accepted cases in detail, did show these main points. Two of the cases occurred in the United States of America and two in Norway. There were three male patients (aged 33, 34, and 46 years) and one female patient (aged 56 years). All showed a prodromal period of upper respiratory infection and all died within 30 to 60 hours of the onset of neurological features. The main differences from the 10 cases discussed above are in the cerebrospinal fluid findings and in the distribution of lesions. The two cases in which cerebrospinal fluid cell counts are recorded showed counts reaching 5,000 and 1,600 per c.mm. with 100% and 85% polymorphs. Some rather equivocal findings of pyogenic cocci in the tissues of the former leave doubts as to its true nature. In addition in two of the cases the white matter of the cerebral hemispheres was spared and lesions occurred only in and below the mid-brain.

As regards the differential diagnosis, acute haemorrhagic leuco-encephalitis has to be distinguished from other causes of multiple petechial haemorrhages and other causes of multiple foci of demyelination. Once microscopic sections are available the combination of haemorrhages, demyelination, and fibrin impregnation in and around the vessel walls leaves little doubt of the nature of the disease. The closest mimicry of the naked-eye lesions is sometimes seen in cerebral fat embolism in which haemorrhages may be limited to the white matter. Other forms of brain purpura usually involve grey and white matter alike.

The aetiology of the disease and the pathogenesis of the lesions form an interesting speculation. The main aetiological possibilities to be considered are (1) invasion of the brain tissue by bacteria or virus, (2) direct action of circulating toxins on the brain tissue, and (3) acquired sensitivity of the brain tissue to a circulating antigen. Bacteria have not been demonstrated in any of these cases and search for a virus has not been reported. In Case 3, however, of the present series some cerebral tissue was obtained immediately after death and two injections of this material were given into the thalamic areas of a rhesus monkey. The monkey showed no abnormal signs apart from slight pyrexia for two days. It was killed 31 days after the first injection but the brain and cord showed no evidence of disease. Passage was not carried out. In addition the structural lesions (particularly the vascular disruption and the polymorphonuclear exudate) and the distribution of the lesions are unlike the findings in any known virus disease. The possibility of direct toxic action is supported

by Hurst's observation (1940) that chronic cyanide poisoning in monkeys could lead to focal demyelination, but in none of the cases of acute haemorrhagic leuco-encephalitis has there been evidence of any direct toxic action of this kind. The third possibility—acquired sensitivity to a circulating antigen—has certain points in its favour. The frequent onset of the brain lesions some days after a respiratory infection is in keeping with the time interval observed with other hypersensitivity states. In particular the lesions are similar to those seen in arsphenamine encephalitis. Russell (1937) described three cases of this condition and drew attention to the evidence suggesting that it was a sensitization phenomenon. More recently Cavanagh (1953) has described somewhat similar lesions in a patient who died with evidence of hypersensitivity following administration of streptomycin and *para*-amino-salicylic acid. Furthermore the vascular lesion resembles closely that occurring in polyarteritis nodosa and producible experimentally in animals by the repeated injection of foreign protein (Klinge, 1929), while the demyelination can be imitated experimentally in animals by multiple injections of emulsions of brain tissue, with or without certain adjuvants (Rivers, Sprunt, and Berry, 1933 ; Rivers and Schwentker, 1935 ; Kabat, Wolf, and Bezer, 1947 ; Lumsden, 1949). The dangers inherent in assuming that similar histological lesions are of similar pathogenesis are well known: nevertheless this similarity of the lesions in the human disease to experimental hypersensitivity lesions and to lesions occurring in drug sensitization is the only positive guide to aetiology in this instance. Clearly, more information is needed on many aspects of the problem, but at present such evidence as there is suggests that the lesions result from hypersensitivity of the brain tissue to a circulating antigen acquired during some upper respiratory infection.

Summary

Three new cases of acute haemorrhagic leuco-encephalitis are described, bringing the total of recorded cases up to 10. The disease occurs predominantly in young adult males.

The encephalitic stage, which is rapidly fatal, is often preceded by a prodromal period of upper respiratory infection.

Polymorphonuclear leucocytosis in the blood and cerebrospinal fluid is the most striking feature of the clinical pathology. The brain lesions are limited to the white matter and are often maximal in the centrum ovale of the hemispheres. They take the form of capillary haemorrhages, polymorphonuclear and serous exudate, fibrin impregnation of vessel walls, capillary plugging, focal demyelination, and microglial proliferation.

The nature of the lesion is uncertain. Search for a causative virus in material from one case was negative. There is some evidence that the lesions may be associated with acquired sensitivity of the brain tissue to a circulating antigen.

I wish to thank Mr. Wylie McKissock, neurosurgeon to St. George's Hospital, for permission to make use of his case records, and Dr. F. O. MacCallum of the Central Public Health Laboratory for carrying out the animal inoculations with material from Case 3.

APPENDIX

Summaries of Case Histories and Post-mortem Findings

Case 1.—A man, aged 28 years, a radio-operator (aircraft), 12 days before admission to hospital developed a feverish cold which lasted four days. He returned to work but six days later became suddenly severely ill with vomiting, headache, and fever. On admission two days later he was semi-conscious, aphasic, and incontinent. Neurological examination showed a small haemorrhage in the right fundus, equal but sluggishly reacting pupils, and right upper motor neuron facial weakness. The tendon reflexes were increased on the right side and the plantar response was extensor on the right, flexor on the left. A radiograph of the skull showed a slight pineal shift to the right. A left temporal-lobe abscess was suspected but exploration proved negative. Ventriculography showed depression of the body and anterior horn of the left lateral ventricle. Cerebrospinal fluid obtained from the right lateral ventricle was under increased pressure and gave a white cell count of 220 per c.mm. (polymorphs 84%) and protein level of 80 mg. per 100 ml. He gradually became more deeply comatose and died four days from the onset of neurological signs.

At post-mortem examination there was evidence of acute bronchitis but otherwise the findings were limited to the brain and are summarized in Table III. Microscopic examination showed widespread haemorrhagic, exudative, and demyelinating lesions of the types described in the text above. Fibrin infiltration in and around the vessel walls was a conspicuous feature.

Case 2.—A boy, aged 2 years 10 months, was healthy until July 19, 1952, when he developed cough and hoarseness. Two days later he became restless and flushed and vomited repeatedly. That evening he developed left-sided convulsions and was admitted to hospital. The temperature was 104.6° F. and he had a left hemiplegia and injected tonsils. A white blood count gave 30,000 per c.mm. (polymorphs 81%). The ears were normal. He was given penicillin and his

temperature fell to normal, but coma and hemiplegia persisted and he was transferred to the neurosurgical centre on July 24 as a possible case of brain abscess. Neurological examination there showed response to painful stimuli only. There was moderate neck rigidity but Kernig's sign was negative. The eyes deviated to the left and upwards, the left pupil larger than the right, the right pupil only reacting to light; the fundi were normal. Other cranial nerve functions were normal. There was paralysis of the left arm and leg with increased tone; abdominal reflexes were present but reduced on the left; the right plantar response was flexor, the left extensor. The cerebrospinal fluid at first (July 21) showed no abnormality and was not re-examined. Ventriculography (July 24) showed some displacement of the ventricles to the left but needling the right hemisphere did not reveal an abscess. The depth of coma increased and he died on July 27.

Post-mortem examination revealed extensive pulmonary oedema with patchy bronchopneumonic consolidation. The brain showed bulging of the right temporal region and on sectioning there was ill-defined, pinkish-grey discoloration of the white matter in this area. Close inspection showed many minute haemorrhagic points in the white matter of both hemispheres. Microscopic examination of the discoloured area showed haemorrhages varying from minute groups of red cells up to areas about 3 mm. in diameter. Many of the haemorrhages had a perivascular distribution, and many of the venules and arterioles showed fibrin impregnation of their walls. Cellular exudate was scanty, but here and there the vessels were surrounded by cuffs of polymorphs and mononuclear cells. Extensive demyelination was present in the discoloured area and many smaller foci of demyelination occurred in adjacent areas of white matter and in the right centrum semi-ovale. The mid-brain, brain-stem, and cerebellum were not involved.

Case 3.—A man, aged 31 years, was employed as a cost accountant. On December 24, 1952, he developed a severe cough and vomited twice. The cough continued and on December 27 he had severe frontal headache with further vomiting. Two days later he complained of left earache, and next day he became drowsy and later unable to recognize his family. Following admission to a local hospital where lumbar puncture was performed (cerebrospinal fluid reported as "cloudy, protein 270 mg. per 100 ml.") he was transferred to the neurosurgical centre as a case of brain abscess on December 31. He was then deeply comatose, and examination of the nervous system gave the following positive findings: extensor spasms of upper limbs on painful stimulation; considerable neck rigidity; pupils contracted and unreactive; limbs extended and spastic but reflexes present and equal; abdominal reflexes absent; right plantar response flexor, left extensor. There was no evidence of otitis, and other systems proved normal. Ventriculography showed a normal ventricular system and the ventricular cerebrospinal fluid contained 150 cells per c.mm. (polymorphs 70%) and 140 mg. protein per 100 ml. His temperature fluctuated from 99° F. to 101.6° F. In the early part of his illness he was treated with penicillin and later with streptomycin, but he never regained consciousness and he died on January 3, 1953, at 11 a.m., five days after the onset of signs of involvement of the nervous system.

At post-mortem examination there was extensive bronchitis and bronchiolitis with patchy bronchopneumonic consolidation of the lower lobes of the lungs. Other positive findings were limited to the brain and are summarized in Table III. Microscopic examination of the brain showed all the types of lesion described above.

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"SERTOLI" AND "LEYDIG" CELLS IN RELATION TO OVARIAN TUMOURS

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The group of tumours termed arrhenoblastomas was separated from the general body of ovarian tumours by Meyer (1915, 1930, 1931). Barzilai (1949) defines the arrhenoblastoma, in conformity with Meyer's views, as "an ovarian tumour the cytoarchitecture of which almost exacly duplicates that seen in the different stages of male gonadogenesis." Thus, although many of these tumours have a masculinizing or defeminizing effect on the host this is not essential for the diagnosis. Descriptions of about 122 of these tumours have been published (Javert and Finn, 1951). Three types of tumour pattern are recognized: (1) a highly differentiated pattern composed of well-developed tubules lined by cubical or columnar cells with basal nuclei, closely resembling Sertoli cells and the late developmental stages of gonadogenesis, as seen in the undescended testis; (2) one in which the tumour looks like a fibromatous or sarcomatous neoplasm; and (3) a pattern intermediate between these two extremes. Barzilai (1949) and Novak (1952) have drawn attention to the large polyhedral eosinophilic cells which frequently lie in the stroma of the tumours and resemble Leydig cells. The work of Sternberg (1949) and of Teilum (1949) allows the separation from this heterogeneous group of two distinct entities (*a*) "Leydig" cell tumours and (*b*) "Sertoli" cell tumours; a third form of tumour also exists in which both "Sertoli" cells and "Leydig" cells occur.

In this paper it is proposed to describe and discuss two tumours, which are morphologically arrhenoblastomas, in the light of these newer concepts, and also a case of bilateral Leydig cell tumour, or hyperplasia, with masculinization.

Case Reports

Case 1.—E. J., a married woman of 64, had menstruated irregularly from the age of 17 until her menopause at 50 years. There had been no pregnancies. For the last 14 years there had been facial hirsuties, increasing recently, and on admission she had a beard on the chin and lips. The pubic and axillary hair were feminine. Her voice had become masculine in the last 12 years. Her breasts were normal apart from a rubbery swelling ½ in. in diameter in one of them. She was obese. Her blood pressure was 200/130 mm. Hg. The vulva and vagina were atrophic. A radiograph of the sella turcica suggested no significant abnormality. On rectal examination a pelvic tumour was felt on the left side. The excretion of ketosteroids was 6.9 mg./24 hrs. (normal 8–17 mg./24 hrs.), and more than 19 mouse units of gonadotrophin were excreted in 24 hours (normal 12–25 mouse units/24 hrs., see Table I). The uterus and adnexae were removed. The patient was seen again 15 months later. There was no significant change clinically, the facial hirsuties persisting and obesity increasing, and the daily excretion of ketosteroids and gonadotrophins remained substantially the same as before the operation.

Macroscopic Appearances.—The uterus was lobular and enlarged by a number of fibroids. The ovaries were slightly enlarged, corresponding in size to those found in the child-bearing period; they contained small cysts and corpora albicantia.

Microscopic Appearances.—The ovaries are slightly enlarged and contain corpora albicantia and germinal inclusion cysts. The ovarian stroma is well developed and composed of intertwining bundles of connective tissue. In the interstices of the stroma are groups of cells with eosinophilic cytoplasm (Fig. 1). These are usually polygonal but in places the corners of the cells are rounded and in others drawn out giving a rather stellate appearance to the cells. The nuclei are round, each having a dense nuclear membrane, a well-defined but light chromatin pattern, and usually a nucleolus. These groups of cells are essentially similar to other groups present in unusually large numbers at the hilum of the ovary in close apposition to non-myelinated nerves and blood vessels, with the possible exception that the nuclei of the hilar cells are denser (Fig. 2).

The cytoplasm of the cells of both groups presents certain features: (1) Some cells possess brown pigment granules (Fig. 3). (2) Some of the cells at the hilum contain small bodies resembling Reinke crystalloids (Fig. 4) as seen in the interstitial cells of

TABLE I
DAILY URINARY EXCRETION OF HORMONES

	Normal Range	Case 1		Case 2		
		Before Operation	15 Months after Operation	Before Operation	10 Days after Operation	2 Months after Operation
17-Ketosteroids	8–17	6·9 mg.	6·6 mg.	17·4 mg.	11·3 mg.	19·1 mg.
Gonadotrophins (F.S.H.) ..	12–25*	More than 19 M.U.	More than 2∿ M.U.	4 M.U.	Less than 8 M.U.	5 M.U.
Pregnanediol				Merely a trace		

* The normal range in the child-bearing period, except just before ovulation.

the testis. (3) Many cells of both groups give a positive reaction with Vines stain more common in the groups at the hilum than in those at the substance of the ovary. The positively reacting material is granular. (4) Some cells of both groups give a positive P.A.S. reaction. This does not occur so frequently as the positive Vines reaction and may be related to the presence of the brown pigment. (5) The cytoplasm of both groups of cells stains faintly with Sudan IV and some have, in addition, granules which stain more deeply. In the stroma there are occasional clusters of sudanophilic granules, but it is difficult to be sure whether these are in the stromal cells or in isolated eosinophilic cells. (6) The cytoplasmic granules stain deeply with phosphotungstic acid haematoxylin.

The relationship of the groups of cells within the ovary to the ovarian stroma is very intimate, collagen fibres (as shown by Van Gieson's stain, Heidenhain's azan, and silver impregnation) penetrating between the individual cells. The impression is given that these eosinophilic cells are derived from the stromal cells, but the evidence is not conclusive and it may be that the stroma is reacting to the presence of these cells as it does in secondary carcinoma of the Krukenberg type.

Case 2.—C. T., an unmarried girl of 18 years, had had four menstrual periods since her menarche at 16. There was facial hirsuties necessitating frequent shaving, a small amount of hair round the nipples, and marked hypertrichosis of legs and back. Pubic hair was of masculine distribution. The clitoris was enlarged. The body outline was masculine, but the voice feminine. The blood pressure was 135/70 mm. Hg; 17-ketosteroid excretion was 17.4 mg./24 hrs. (normal 8–17 mg./24 hrs.), 4 mouse units of gonadotrophin were excreted per day and only a trace of pregnanediol. On vaginal examination a cystic mass was found to the right of the uterus. At laparotomy this mass, which proved to be a right ovarian tumour, was removed.

Two months after excision of the tumour, she menstruated and continued to do so regularly at 30-day intervals, while basal temperature studies showed evidence of ovulation. Her bust measurement increased by 2½ inches in 10 months and her figure became feminine. Although she continued to shave once a fortnight her psyche had changed and she was able to cry at a sad radio story! A month after the operation, the 17-ketosteroid excretion was 11.3 mg./24 hrs., and the gonadotrophin less than 8 mouse units /24 hrs. Two months later the corresponding figures were 19.1 and 5 respectively (Table I).

Macroscopic Appearance of the Tumour.—This was a yellow mass (6 × 4 × 1 cm.) with central cystic cavities. The surrounding solid areas were lobular and flecked with yellow.

Microscopic Description.—The tumour has no well-marked pattern but consists of sheets of fairly large cells showing a slight tendency to aggregate into clumps, the intervening connective tissue appearing to be oedematous. Small areas of haemorrhage are also present and a few large sinuses—possibly lymphatics. The main cells of the tumour have fairly large nuclei each having a well-marked nuclear membrane and a light chromatin pattern in which frequently a nucleolus can be seen. The cytoplasm of these cells is faintly eosinophilic and the cell margins are ill defined. In one place these cells are arranged in a radial manner giving rise to acinar structures (Fig. 5). Scattered through the tumour, singly or in groups, are cells with markedly eosinophilic cytoplasm (Fig. 6). They are usually polygonal or rounded, but occasionally elongated, and have well-defined cell boundaries. The nuclei of these cells are similar to those of the main cells of the tumour but tend to be larger each having a nucleolus, and sometimes more than one nucleolus. These cells present the following features: (1) Most, probably all, of these cells give a positive reaction with Vines stain. (2) A very few cells give a positive P.A.S. reaction. (3) The cytoplasm is finely vacuolated. Some of the larger of these cells have taken on a xanthomatous appearance. Much fat is present in the cytoplasm of these eosinophilic cells but one cannot be sure that it is confined to these cells only. (4) Many of the eosinophilic cells, possibly all, stain darkly with phosphotungstic acid haematoxylin. (5) No brown pigment or Reinke crystalloids are present (cf. Leydig cells of premature testis).

Reticulin fibres sometimes surround these groups of eosinophilic cells and at others penetrate the groups separating the individual cells. A comparison of the eosinophilic cells in these two cases with ovarian hilar cells and testicular Leydig cells is shown in Table II. It can be seen that the eosinophilic cells of the ovarian lesions are very similar, their differences being of the same type and order as the differ-

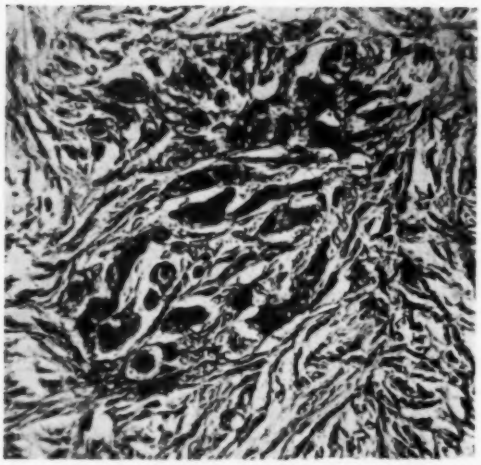

FIG. 1.—Case 1: A group of "Leydig" cells in the stroma of the ovary (Vines stain ×400).

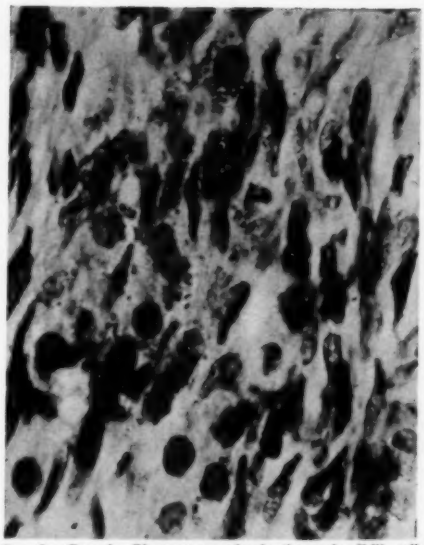

FIG. 3.—Case 1: Pigment granules in the eosinophilic cells of the ovarian stroma (haematoxylin and eosin ×1,000).

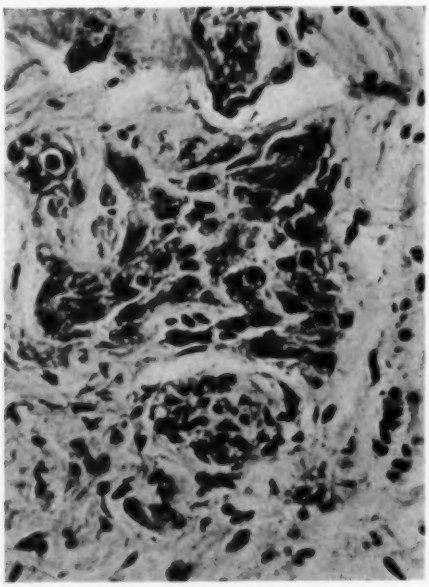

FIG. 2.—Case 1: A group of "Leydig" cells at the ovarian hilum. Note the nerve below and the blood vessel above (haematoxylin and eosin ×400).

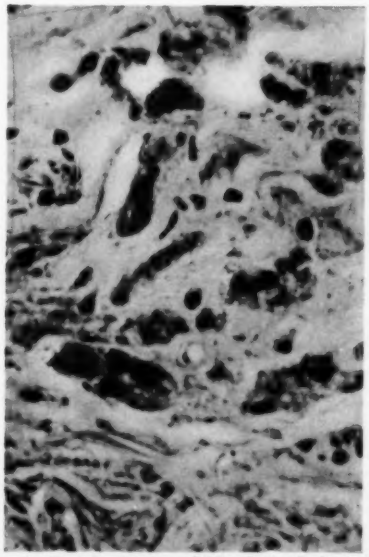

FIG. 4.—Case 1: A group of "Leydig" cells from the ovarian hilum. The cell above and to the left of the centre of the field contains a long, fairly broad, rod-shaped crystalloid of Reink: which crosses in front of the nucleus and occupies much of the cell (Masson's trichrome stain ×500).

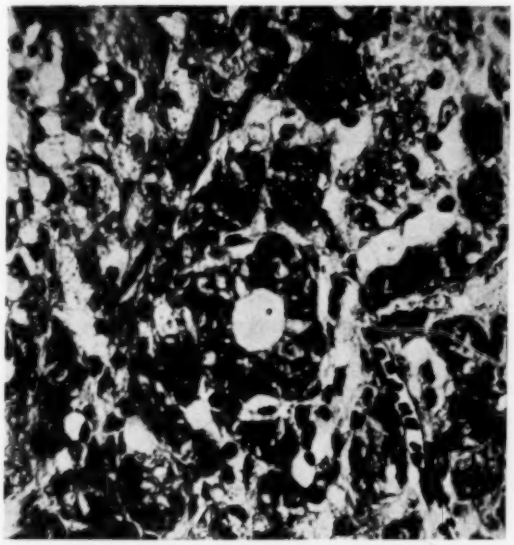

FIG. 5.—Case 2: A group of the main cells of the tumour forming an acinus (haematoxylin and eosin ×400).

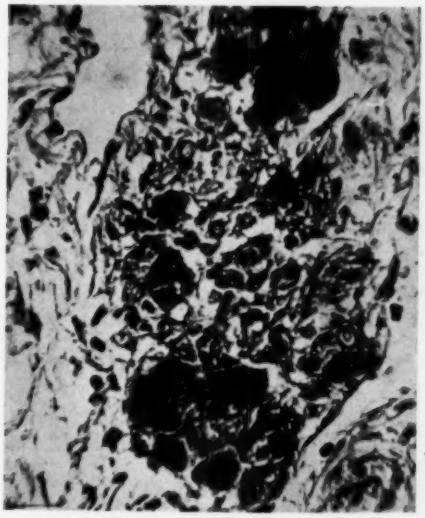

FIG. 8.—Case 3: The dark cells are eosinophilic "Leydig" cells in the band of tissue connecting the left ovary to the broad ligament. Note the close relationship of these cells to a blood vessel (Vines stain ×400).

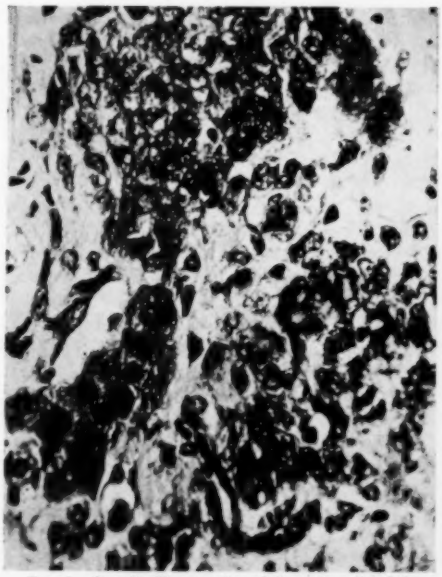

FIG. 6.—Case 2: The margins of the main cells of the tumour are ill-defined and the nuclei are irregularly arranged. The eosinophilic cells form groups having an orderly arrangement. The cytoplasm of these cells is finely vacuolated (haematoxylin and eosin ×400).

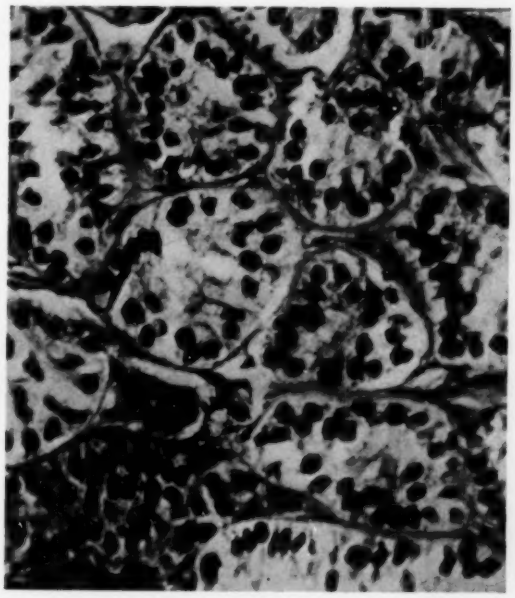

FIG. 7.—Case 3: The boundaries of the cells cannot be defined and the cytoplasmic tendrils intermingle in the lumen. There is a tendency for the acini to be lined by more than one layer of cells (haematoxylin and eosin ×400).

TABLE II

A COMPARISON OF THE CYTOPLASM OF "LEYDIG" CELLS

	Adult Testis	Infant Testis	Ovarian Hilar Cells	Case 1		Case 2	Case 3
				Cells at Hilum	Cells in Stroma		
Pigment ..	+	0	+	+	+	0	+
Reinke crystalloids	+	0	+	+	0	0	0
Acidophil bodies ..	+	0	+	0	0	+	+
Vines reaction	±	Patchy	+	++	++	+	+
P.A.S. ..		0		+	+	A few cells	A few cells
Vacuolation	+	+	+	+	+	+	+
Fat (Sudan IV)	+	+	+	+	+	+	+
Phosphotungstic acid haematoxylin	±	A few cells	+	+	+	+	+

ences between the Leydig cells of an infant's testis and those of an adult man.

Case 3.—Mrs. G., a married woman of 62, who had never menstruated, had for two months complained of attacks of bearing-down pain in the left groin. The last attack occurred the week before admission. On examination a mass was found in the hypogastrium extending from the pelvis and on vaginal examination this was thought to arise from the right ovary. The cervix could not be found. On opening the abdomen a large tumour, about 14 cm. in diameter, was found on the right side. The left ovary lay at the pelvic brim together with a small cyst which had undergone torsion. No uterus was found but ribbons of muscular tissue running laterally on each side and arising from a structure resembling the broad ligament seemed to represent hypoplastic uterine horns.

The cut surface of the tumour was fawn and crossed by strands of fibrous tissue.

Microscopic Examination.—The tumour consists of small acini and tubules lined by cells with clear cytoplasm and basal nuclei. The cells lining the tubules are usually single layered but sometimes form several layers (Fig. 7). The cytoplasm trails off towards the lumen as fine tendrils which intertwine. The boundaries of the individual cells can rarely be determined, giving a syncytial appearance to the epithelium. The tubules and acini are separated by connective tissue strands, which are in places hyalinized and in others loose and oedematous. The epithelium of a number of tubules contains fine droplets of fat. In the angles between some tubules a few "interstitial" cells can sometimes be found.

The ovary on the left is composed of typical ovarian tissue and also contains a nodule having a similar structure to the right-sided tumour. In the band of tissue connecting the left ovary to the broad ligament are groups of eosinophilic cells similar to those lying between some of the tubules (Fig. 8). The nuclei of these cells are pale with dense nuclear membranes and each has a nucleolus. The cytoplasm is vacuolated and contains fat, it gives a positive Vines reaction, granules of brown pigment are present in some cells, and some cells contain P.A.S.-positive granules. The cytoplasm stains deeply with phosphotungstic acid haematoxylin. No Reinke crystals are present, but irregular masses of eosinophilic material can be seen in the cytoplasm with Masson's trichrome stain. The cells lie closely adjacent to blood vessels. Generally the cells are separated from each other by reticulin fibrils but in places fibres surround a group of cells. Thus these cells conform morphologically to hilus (or Leydig cells) both in site and cytology. A cytological comparison between these cells and the eosinophilic cells in Cases 1 and 2 is shown in the Table II.

Discussion

The "Leydig" Cells of the Ovary.—Sternberg (1949) has reviewed the present state of our knowledge of extra-testicular Leydig cells which, in women, he terms hilus cells, since they are found at the hilum of the ovary. They occur in at least 80% of ovaries. These cells are eosinophilic and form unencapsulated groups lying close to, or actually intermingling with, non-myelinated nerve trunks and also near to small blood vessels. These features are seen in extra-glandular groups of cells in Case 1 (Fig. 2). The cytoplasm of the cells is variable; it may contain fat, brown pigment granules, and Reinke crystalloids, and the mitochondria stain darkly with phosphotungstic acid haematoxylin. Reinke crystalloids are often difficult to find, but they may be associated with spheroidal hyaline acidophil bodies having the same staining reaction. The nuclei vary a little, but often contain one or more nucleoli and the cells are very occasionally multinucleated. These features are present in testicular Leydig cells and in this organ variations in numbers of cells and cytoplasmic contents also occur with age. The cells are very conspicuous in the testes of the male foetus and of premature infants, but after birth become scanty until puberty. In the infant pigment is absent but fat is present. In the adult the lipid content of the cells varies with their functional activity (Teilum, 1950). In Table II the features of the eosinophilic cells described in the tumours described here is compared with that of the testicular Leydig cells. Bearing in mind the variability of Leydig cells the morphological correspondence of the ovarian cells to the testicular ones is good except perhaps with regard to the occurrence of Reinke crystals. Others have found this feature difficult to demonstrate (Schiller, 1950).

The cells at the ovarian hila in Case 1 are morphologically identical with hilus cells and the intra-ovarian eosinophilic cells are similar to these but lack Reinke crystalloids. Brown pigment in

the cytoplasm of intra-ovarian cells distinguishes them from lutein cells. The normal level of excretion of 17-ketosteroids renders the origin of the "Leydig" cells from ectopic adrenal tissue unlikely.

It may be that Case 1 should more properly be termed one of hyperplasia rather than of a neoplasm. In favour of the condition being hyperplastic is its bilateral situation, but against this there is the apparent invasion of the ovarian stroma by hilus cells unless it is supposed that the intra-ovarian Leydig cells arise by metamorphosis of the stroma. In the two cases of hilus-cell hyperplasia with masculinization reported by Sternberg (1949) the cells were confined to the hilum.

Case 1 bears some resemblance to the cases of Geist and Gaines (1942) and Rottino and McGrath (1943) in which luteinization of the ovaries is described in association with masculinization. The criteria used by these workers in determining the lutein nature of the ovarian change are uncertain and hormone analyses are lacking.

The general architecture of the tumour in Case 2 is of an arrhenoblastoma of Meyer's intermediate class. The eosinophilic cells resemble the Leydig cells of the foetal testis and also the hilar cells of the infantile ovary, but lack the pigment and crystalloids of the adult cells. Berger (1942) reported a virilizing tumour of the mesovarium in a woman of 50. This he considered to be of "Leydig" cell type although Reinke crystalloids and cytoplasmic pigment were not present. Although lutein cells have many of the features of hilar cells (including a positive Vines reaction) only a trace of pregnanediol was excreted, thus rendering it unlikely that the eosinophilic cells of this tumour were lutein. A positive Vines reaction is also given by cells in the foetal adrenal cortex, in hyperplastic adrenal cortex and in cortical adrenal tumours (Broster and Vines, 1933; Zinsser and Zinsser, 1951). Thus the eosinophilic cells of this tumour might arise from ectopic adrenal tissue near, or in, the ovary, but in adrenal hyperplasia and masculinizing adrenal tumours the excretion of 17-ketosteroids is usually greatly increased. Normal excretion can occur, although very rarely (Escamilla, 1949; Kepler and Mason, 1947; Venning and Browne, 1947). The excretion of 17-ketosteroids in the two patients of Sternberg (1949) with hilar cell tumours was normal; in the patient of Waugh, Venning, and McEachern (1949) it was moderately increased but the left adrenal was also enlarged. The patient of Sachs and Spiro (1951) had a normal 17-ketosteroid excretion but this may have been due to coincidental malignant cachexia. All these tumours were identifiable as "Leydig" cell tumours by their site and by the presence of Reinke crystalloids in the cytoplasm of the constituent cells. Thus in morphological features and on the grounds of hormone excretion the eosinophilic cells of Case 2 are compatible with a "Leydig" cell origin and are not lutein or adrenal in nature.

Sternberg (1949) considered that his two cases of hilus-cell tumour, and that of Berger (1942) and another mentioned in an addendum to his paper, to be the only hilus-cell tumours reported up to then. The above analysis suggests that his view is too limited and that eosinophilic cells seen in many arrhenoblastomas and described as Leydig cells (Brentnall, 1945; Teilum, 1949) without further specification of their properties are, in fact, "Leydig" (or hilus) cells. Besides arrhenoblastomas, other groups of masculinizing ovarian tumours occur associated with eosinophilic cells; these have been termed "virilizing lipoid cell tumours" (Barzilai, 1949) and "adrenocorticoid tumours" (Herbut, 1953). It seems probable that some tumours such as that of Merivale and Forman (1951), which had a high urinary output of 17-ketosteroids, are adrenal rather than "Leydig" in type.

The Nature of the Tubular Adenomas.—The characteristic features of the epithelium lining the tubules in Case 3 are (i) the palisade arrangement of the cells, (ii) the vacuolated fibrillary cytoplasm, (iii) the ill-defined cell boundaries with interlacing cell processes (see Fig. 7), giving a syncytial appearance, and (iv) the presence of sudanophilic granules. These features are found in the Sertoli cells in atrophic testes (Innes, 1942; Stalker and Hendry, 1952).

Pick (1905) recognized the morphological similarity between these tumours and testicular tissue, but this similarity may also extend to their function. Pick's patient was a woman of 34 suffering from metrorrhagia and had no masculinizing signs. Likewise the patients described by Schickele (1907), Grevle (1936), Henderson (1942), and Dougal (1945) had menorrhagia or metrorrhagia. In the case recorded by Burslem, Langley, and Woodcock (1953) the patient had irregular vaginal bleeding associated with cystic hyperplasia of the endometrium. These features suggest that such tumours are oestrogenic rather than androgenic. Similar tumours occur in the testis and, although they usually have no hormonal effect (Stalker and Hendry, 1952), Teilum (1946) has described such a tumour in a man of 53 years associated with gynaecomastia and increased

oestrogen excretion. Likewise feminizing testicular tumours of similar morphology have been described in dogs by Innes (1942) and by Huggins and Moulder (1945).

Although these tubular (or Sertoli cell) tumours of the ovary are often feminizing they are occasionally masculinizing, as in Meyer's (1930) Case 2 and in Phelan's (1934) patient. Sometimes they are hormonally indifferent and such tumours are often associated with rudimentary sex organs or their congenital absence (Novak, 1943; Jolles and Gleave, 1945; Goldberg and Maxwell, 1947). My Case 3 falls into this group. It is interesting to note that of the 17 cases of tubular adenoma of the testis which Stalker and Hendry (1952) have collected from the literature, 11 occurred in pseudohermaphrodites.

The Histogenesis of Sertoli Cell Tumours.—Whereas "Leydig" cells appear to be a very frequent, if not constant, component of the ovary Sertoli cells are normally confined to the testis. It must however be remembered that, whatever the details of embryogenesis, Sertoli and granulosa cells have the same *Anlage* (Gillman, 1948) and cannot be distinguished until about the 15 mm. stage in the human foetus (Norris, 1938). Thus it is evidently possible under abnormal conditions for the precursor of the definitive granulosa cell to mature along an alternative line and give rise to a Sertoli cell. That this can happen is shown by the case of Burslem *et al.* (1953) in which both a Sertoli cell tumour and a granulosa cell tumour merge. Meyer (1930) described two tumours (Cases 4 and 7) showing a similar merging.

Interference with differentiation may cause an apparent reversal of sex (Popoff, 1930; Berger, 1945). Dantchakoff (1936) has produced the full feminization of genetically male birds by introducing oestrone dissolved in oil into the allantoic cavity on the fourth day of incubation. After hatching these developed as intersexes. Dantchakoff (1937) has also produced masculinization of guinea-pig embryos by use of testosterone propionate. Lillie (1916, 1917) showed that when the foetal membranes of male and female calf embryos become united *in utero* so that the circulations are in continuity the female partner becomes greatly modified in the male direction forming an intersex (freemartin) with testes and male ducts. Remembering that many of these Sertoli cell tumours both in men and women are associated with congenital anomalies of the genital tract the possibility that the tumour itself is a result of disturbed gonadogenesis becomes likely, the disturbing factor possibly being in the maternal hormone system. Thus Sertoli cell tumours of this class should be considered as congenital malformations, or hamartomas, rather than blastomas. This concept may, perhaps, apply to other Sertoli cell tumours in which disturbance of development has not involved the rest of the genital tract.

Conclusion

Accepting the concept of the term arrhenoblastoma as introduced by Meyer and crystallized in the definition of Barzilai (1949) as "an ovarian tumour the cytoarchitecture of which almost exactly duplicates that seen in the different stages of male gonadogenesis," it is possible to subdivide them into at least three groups: (*a*) those in which "Leydig" cells are a conspicuous feature, (*b*) those in which "Sertoli" cells are conspicuous, and (*c*) a mixed group in which both "Sertoli" and "Leydig" cells occur. Such a classification does not include the virilizing lipid-cell tumours, nor the masculinizing tumours with a "sarcomatous" pattern.

Summary

Two patients with masculinization of ovarian origin are described. The first had bilateral tumours, or hyperplasia, of ovarian hilus (extratesticular Leydig) cells and the second had an arrhenoblastoma in which "Leydig" cells were a conspicuous feature.

A third patient is described who had bilateral ovarian tumours of tubular, "Sertoli" cell, type associated with congenital malformation of the genital tract.

The relationship of these tumours to the classification and histogenesis of arrhenoblastomas is discussed.

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SPECIFICITY OF AUTO-ANTIBODIES IN ACQUIRED HAEMOLYTIC ANAEMIA

BY

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Until recently it has been assumed that the auto-antibodies found in acquired haemolytic anaemia are non-specific; that is to say they react with all types of human red cells and are unrelated to any known blood group antibodies. However, Weiner, Battey, Cleghorn, Marson, and Meynell (1953) have described a patient in whom the auto-antibody had the specificity anti-e, the patient's red cells having the genotype CDe/CDe: the same phenomenon had previously been suspected in a patient investigated by Dr. Ruth Sanger (personal communication).

In the course of investigating a severe case of acquired haemolytic anaemia (Case 1), we noted that the patient's serum strongly agglutinated red cells of the genotypes CDe/CDe and cde/cde but not those of cDE/cDE. Further investigation showed that the patient's serum contained anti-C and anti-e, despite the fact that the patient's genotype was CDe/cde. This observation led to the re-investigation of nine other cases of acquired haemolytic anaemia whose warm auto-antibodies had been considered to be non-specific; the findings are described briefly in the present report.

Materials and Methods

The red cells from each of the 10 patients in whom acquired haemolytic anaemia had been diagnosed gave a positive direct Coombs test. In each case when the red cells were tested against serial dilutions of a highly potent antiglobulin serum a prozone was observed. As with red cells sensitized by anti-D, the agglutination was readily inhibited by adding small amounts of human gamma-globulin to the antiglobulin serum (Dacie, 1951). The patients' sera were known to contain cold antibodies in very low titre only.

In eight of the 10 cases, the Rh genotype of the patients' cells had been determined; the remaining two patients were no longer available. All 10 patients had received blood transfusions during the course of their illness.

The specificities of the antibodies in the patients' sera were investigated, using red cells from normal persons. The sera were tested for their ability to agglutinate trypsinized red cells (at 37° C.) and to sensitize red cells to an antiglobulin serum (at 37° C.). When possible, eluates were prepared from the patients' red cells by the method of Kidd (1949), and were then tested for the presence of antibodies. In addition, the sera and the eluates were absorbed with red cells of different genotypes and then tested against a panel of cells.

In these tests, red cells of the Rh genotypes CDe/CDe, cDE/cDE, -D-/-D- and cde/cde were used; their F f constitution was not known with certainty, except in the case of one Rh-negative sample of the rare genotype cdeF/cdef. The reactions of all these cells with the following antisera had been determined: anti-M, anti-N, anti-S, anti-P, anti-Le^a, anti-Le^b, anti-Lu^a, anti-K, and anti-Fy^a; in some cases their reactions with anti-Jk^a and anti-Jk^b had also been determined. Cells from donors of group O were used in the majority of tests. In some cases, where the patient was group A, the serum was tested with red cells of group A and of group O. The -D-/-D- cells were obtained from the subject described by Race, Sanger, and Selwyn (1951).

Results

In only one case (Case 1, Ew., already mentioned above) did the patient's serum fail completely to react with cells of the genotypes cDE/cDE and -D-/-D-. This patient's serum nevertheless agglutinated her own cells and other cells containing the antigens e or C; it appeared to contain anti-e and anti-C but no non-specific antibody. That the serum did in fact contain anti-e was confirmed by tests with red-cell samples from 22 persons of known genotype, all of whom lacked the antigen C; five of them were of the genotype cDE/cDE. The serum failed to react only with

those samples which lacked the antigen e, as follows:

		Red Cells e+	Red Cells e−
Reactions with Ew. serum	+	17	0
	−	0	5

The probability of getting such a result by chance is less than 1 in 26,000.

The anti-e could be removed by repeated absorptions with cde/cde cells. After absorption the serum was tested with red-cell samples from 14 persons, all of whom possessed the antigen e; eight of these also possessed the antigen C. The absorbed serum gave the following reactions:

		Red Cells C+	Red Cells C−
Reactions with absorbed Ew. serum	+	8	0
	−	0	6

These results showed that anti-C was present in the serum, since the probability of getting this distribution by chance is 1 in 3,000.

It was concluded that, despite the fact that this patient's own red cells contained the antigens C and (genotype CDe/cde), her serum contained the antibodies anti-C and anti-e. The patient was acutely ill and died of her disease a few days after admission to hospital. Unfortunately an eluate was not prepared from her cells.

In all the other nine cases investigated the patients' sera, at 37° C., reacted to some extent with all the samples of red cells against which they were tested, as well as with the patients' own red cells; that is to say, the sera apparently contained warm non-specific antibodies. However, it was soon obvious that in some cases the reactions were consistently stronger with red cells of a particular genotype, suggesting that specific antibodies were also present. These specific antibodies were of two kinds: those reacting with antigens not present in the patients' own cells ("immune iso-antibodies") and those reacting with antigens present in the patients' own cells ("auto-antibodies").

It was concluded that the immune iso-antibodies had been formed as a result of previous transfusions. These antibodies could be identified as anti-E in three cases and as anti-c plus anti-E in one case. In Case 8, the anti-E was thought to be an immune iso-antibody, although the patient's Rh genotype had not been determined.

The specificity of the auto-antibodies could be investigated most directly by examining an eluate prepared from the patient's own red cells. Eluates were prepared in five cases; they were tested for their ability to agglutinate trypsinized cells and to sensitize cells to an antiglobulin serum both before and after absorption with trypsinized cells and with normal red cells. Some of the findings in three of these cases are shown in Table I.

In Case 2, the unabsorbed eluate reacted more strongly with red cells containing the antigen e, and after absorption with cDE/cDE cells it reacted exclusively with e-positive cells. It was concluded that the eluate contained anti-e. The eluate appeared also to contain non-specific antibody; it reacted with e-negative cells and this activity could be removed by absorption with cells of genotype CDe/CDe, cDE/cDE or cde/cde. However, after one absorption with -D-/-D- cells, the eluate still reacted with cDE/cDE cells, although it no longer reacted with -D-/-D- cells (see Table I). Such behaviour was not consistent with that of a single non-specific antibody but suggested the presence of two components in addition to anti-e.

In Cases 4 and 6, the unabsorbed eluates reacted equally well with red cells of all groups and at first appeared to contain wholly non-specific antibodies. In Case 4, after absorption with cells of genotype CDe/CDe, cDE/cDE or cde/cde, the eluate failed to sensitize cells of any group; however, as in Case 2, the antibody which reacted with these cells was not removed completely by absorption with -D-/-D- cells. The antibody present in the eluate of Case 6 behaved similarly after absorption with -D-/-D- cells (see Table I). The antibodies, other than anti-e, detected in the eluates of Cases 2, 4, and 6, are referred to in the tables as "non-specific antibody plus an unidentified component." The non-specific antibody reacted with, and was absorbed by, red cells of all the genotypes tested; the other component reacted with, and was absorbed by, all types of red cell except -D-/-D-. Neither component appeared to be related to a known blood-group antigen.

In view of the strong sensitization of normal cells by some eluates (such as that of Case 2) in which the presence of anti-e was suspected, an attempt was made to demonstrate that these cells were "blocked," i.e., were no longer agglutinated by anti-e. Unfortunately only a very weak saline-agglutinating anti-e was available and the results were inconclusive.

The findings in all 10 cases are summarized in Table II. It will be noted that in some cases the antibody, both in the serum and the eluate, acted preferably with trypsinized cells, and in a few cases was detectable only with trypsinized cells. For instance, in the eluate of Case 4 specific auto-antibodies were detected using trypsinized cells although none was demonstrable by the indirect

TABLE I

REACTIONS OF NORMAL RED CELLS WITH ELUATES PREPARED FROM THE RED CELLS OF THREE PATIENTS WITH ACQUIRED HAEMOLYTIC ANAEMIA (RESULTS OF INDIRECT COOMBS TEST)

Case No. and Probable Genotype	Eluate after (×) Absorptions	Reactions with Normal Red Cells				Interpretation
		CDe/CDe	cDE/cDE	-D-/-D-	cde/cde	
2. A_1 CDe/CDe	Not absorbed	++++	+±	+±	++++	Anti-e plus non-specific (2)
	CDe/CDe (×4)	0	0	0	0	
	cDE/cDE (×4)	+++	0	0	+++	
	-D-/-D- (×1)	++++	±	0	++++	
	cde/cde (×1)	+++	0	0	++	
	cde/cde (×4)	0	0	0	0	
4. A_2 CDe/cde	Not absorbed	++++	++++	++++	++++	Non-specific (2)
	CDe/CDe (×2)	0	0	0	0	
	cDE/cDE (×2)	0	0	0	0	
	-D-/-D- (×2)	+	±	0	+	
	-D-/-D- (×4)	+	±	0	+	
	cde/cde (×2)	0	0	0	0	
6. A_1 cde/cde	Not absorbed	+++	+++	++	+++	Non-specific (2)
	CDe/CDe (×1)	0	0	0	0	
	cDE/cDE (×1)	0	0	0	0	
	-D-/-D- (×1)	++±	++±	0	++±	

++++ = very strong agglutination. ± = weak agglutination. 0 = negative reaction. Non-specific (2) = non-specific antibody plus an unidentified component.

TABLE II

FINDINGS IN 10 CASES OF ACQUIRED HAEMOLYTIC ANAEMIA

Case	Patient's ABO Group and Probable Rh Genotype	Apparent Specificity of Antibody Components		
		Immune Iso-antibodies	Auto-antibodies	
			Serum	Eluate from Patient's Cells
1. Ew.	B CDe/cde	None	Anti-C + anti-e	....
2. Fo.	A_1 CDe/CDe	Anti-E	Anti-e + ? non-specific	Anti-e + non-specific (2)
3. Fi.	B CDe/cde	,,	Anti-e† + non-specific	....
4. Co.	A_2 CDe/cde	None	Anti-e + non-specific	Anti-e†, anti-D‡ + non-specific (2)
5. Da.	A_1 CDe/cde	,,	Anti-e + non-specific	....
6. Ka.	A_1 cde/cde	,,	....	Non-specific (2)
7. Ho.	A_1 CDe/cDE	,,	Non-specific †	Non-specific
8. Pe.*	B Rh+	Anti-E	,, †	....
9. Al.	A Rh+	None	,, †	Non-specific
10. To.	O CDe/CDe	Anti-c + anti-E	,, ‡	....

.... = not tested. Non-specific (2) = non-specific antibody plus an unidentified component. † = acted preferably with trypsinized cells ‡ = reacted with trypsinized cells only. * It was assumed that this patient lacked the E antigen, although the Rh genotype was not determined

Coombs test. Anti-e was found in eluates obtained early in this patient's illness: anti-D was found in later samples.

Discussion

The majority of patients suffering from acquired haemolytic anaemia form antibodies of the "warm" type: i.e., their serum does not contain abnormal amounts of cold antibody and their red cells, when tested with an antiglobulin serum, react as if sensitized with anti-Rh. All 10 cases described here were of this type.

In the five cases in which the patient formed an auto-antibody reacting with specific antigens, the antibody was found to have specificity within the Rh system: anti-e + anti-C (one case) anti-e + anti-D (one case), anti-e (three cases). It is perhaps remarkable that, since anti-D is formed so readily as an immune iso-antibody, only one of the nine D-positive patients formed anti-D as an auto-antibody. In contrast, anti-e, which is so seldom found as an immune iso-antibody, was formed by five patients as an auto-antibody.

In most cases auto-antibodies without apparent specificity were also present. In three cases such antibodies were found together with an antibody which was absorbed by all types of cells except -D-/-D- cells. This suggests that the -D-/-D- cells lacked an antigen other than c, C, e and E.

Recently, Wiener, Gordon, and Gallop (1953) have concluded that the warm antibodies formed in patients with acquired haemolytic anaemia are directed "against the nucleus of the Rh-Hr substance." Our findings certainly support the idea that in some cases the warm auto-antibodies of acquired haemolytic anaemia may be indistinguishable from immune Rh antibodies.

Summary

An attempt has been made to determine the specificity of the warm auto-antibodies developed by 10 patients with acquired haemolytic anaemia.

In five cases the auto-antibodies were directed against Rh antigens (e, C or D). In nine of the 10 cases auto-antibodies of apparently non-specific nature were present.

Four patients also developed anti-E, probably as the result of previous transfusions.

We should like to thank the physicians of the Postgraduate Medical School of London for permission to investigate patients under their care. Other patients have been investigated through the courtesy of Drs. J. O. M. Edgecombe, E. H. Hempstead, G. B. Hollings, F. Rackow, A. I. Ross, J. Stewart, and G. H. Tovey. We are greatly indebted to Dr. Dorothy M. Parkin and to Dr. J. R. E. Richardson for samples of red cells of rare genotypes, to Dr. E. M. Martland and Dr. J. G. Selwyn for a sample of blood of genotype -D-/-D-, and to Dr. Sheila Worlledge for her help during this investigation.

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AN ACQUIRED COAGULATION DEFECT IN A WOMAN

BY

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A prolonged clotting time is rare in women. Nine cases have been reported, and from Table I, which summarizes these and the present case, it will be seen that all except one occurred within a few months of childbirth. In all, the clinical story was surprisingly similar : all had deep ecchymoses, and most had epistaxis, haematuria, and often effusions into joints and melaena. One patient died of suffocation from haemorrhage into the tongue ; some have continued unchanged for a number of years ; some have slowly recovered, while the present patient recovered in a few months.

Laboratory investigation showed in all a normal bleeding time (except possibly in the present case), a normal platelet count, and a normal tourniquet test except in Madison and Quick's case (1945). The prothrombin time was normal in all cases in which it was performed. The Lee and White coagulation time was long in every case, and varied from half to two hours. In five there was evidence that an inhibitor was present which seemed to act either on the formation of thromboplastin or specifically against the antihaemophilic globulin. In two others there was fragmentary evidence to suggest that the patient's plasma and that from a haemophiliac were not mutually correcting. The present case falls into this group also.

The rarity of these cases, and the interest of the investigation of each case in relation to the then accepted theory of blood coagulation, seem to justify reporting the investigation of a further example of this mixed group, even though the patient's recovery prevented the fullest investigation.

Case History

A married woman, aged 35, had had no relevant illness and gave a history with no haemorrhagic incidents until three months after the birth of her first baby. For the next three months she had a series of large, subcutaneous, spontaneous bruises and an attack of heavy, painless haematuria. These symptoms slowly cleared up and she has remained well for one year up to the present.

The routine investigations are presented in Table II. The bleeding time was considerably longer than the average normal figure in women, but occasionally

TABLE I

SUMMARY OF REPORTED CASES

Author	Age	Following Pregnancy	Clotting Time (min.)	Tourniquet Test	Nature of Defect	Final Outcome
Joules and Macfarlane (1938)	56	No	43–67	–	Not corrected by haemophilic blood	No change in 5 years
Madison and Quick (1945)	30	Yes. 6 months after	17–21	+	?	Asphyxial death from lingual haemorrhage one year later
Chargaff and West (1946)	33	Yes. 7 months after	80–100	–	? Inhibitor	—
Fanti and Nance (1946)	39	Yes. 3 months after	26–36	–	Specific anti-thromboplastin	Continued at least 9 years
Hewlett and Haden (i) (1949)	40	Yes. 3 months after	116	–	?	Almost complete recovery in 2 years; normal pregnancy in this time
(ii)	33	Yes. 13 months after	135		Haemophilic plasma clotting time not corrected by the addition of tenth part of patient's plasma	Improved in 2 years
Heinle *et al.* (1949)	34	Yes. 12 months after		–	An inhibitor producing deficiency of available thromboplastin	6 years. ? Static
Dreskin and Rosenthal (1950)	30	Yes. 3 months after	39–81	–	? Anti-thromboplastinogen	Only clinical recovery in 1 year
Biggs *et al.* (1952)	35	Yes. 8 months after	26–50	Weak positive	Inhibitor of anti-haemophilic globulin	Continued for at least 6 months
Present case ..	35	Yes. 3 months after	15–33	–	? Platelet defect and apparent absence of antihaemophilic globulin	Complete recovery in 4 months

TABLE II
ROUTINE INVESTIGATIONS

Haemoglobin	12·4 g.%
White cells	9,000 per c.mm.
Differential white count	Normal
Platelets	120–300,000 per c.mm.
Ivy bleeding time	3½, 9½, 11½, 9½, 14, >15 min.
Tourniquet test	Negative
*Lee and White coagulation time (in glass)	15–33 min.
Lee and White coagulation time in silicone-treated tubes	5 hours
Quick one-stage prothrombin time	16 seconds (normal)
Merskey two-stage prothrombin time	17 seconds.
*Serum prothrombin time (expressed from Lee and White tubes)	12 seconds
*Prothrombin consumption index	142%
*Serum prothrombin time (expressed from clotted plasma)	14–23 seconds

* These figures were obtained at the beginning of the illness.

such long figures are observed in otherwise normal people (O'Brien, 1950).

The evidence in Table II indicates that the defect is due to the inadequate conversion of prothrombin to thrombin. A normal amount of prothrombin seems to be present and it is able to function normally as indicated by the normal one-stage prothrombin time, but the intrinsic thromboplastin is inefficient. The factors thought to be necessary for the formation of thromboplastin are platelets, factor V, antihaemophilic globulin, factor VII (Koller, Loeliger, and Duckert, 1951), the Christmas factor (Biggs *et al.*, 1952; Biggs, Douglas, and Macfarlane, 1953), and calcium. Any one or more of these factors may be deficient or prevented from working by a specific inhibitor.

Technique

Blood collected by a clean venepuncture was mixed with one tenth part of 3.8% sodium citrate and centrifuged at 1,500 r.p.m. for five minutes and the supernatant pipetted off.

When plasma relatively free of platelets and a platelet suspension was required, siliconed apparatus was used. Slow-spun plasma prepared as above was recentrifuged at 6,000 r.p.m. for 15 minutes and the supernatant called "high-spun plasma." The viscid mass at the bottom of the tube was drained dry by inversion, and emulsified into a volume of saline equal to one-third of the original plasma volume. A homogeneous suspension was not obtained. The brain thromboplastin used was made from acetone-dried human brain and gave normal prothrombin times of 14 to 17 seconds. The calcium chloride solution was M-40. The fibrinogen was a 1% solution in saline of the human fibrinogen prepared by the Lister Institute. The prothrombin consumption index was estimated according to the technique of Mersky (Mersky, 1950). All experiments were carried out with the reacting tubes and solutions in a water-bath at 37° C.

Results

The rate of formation of thrombin in recalcified plasma was watched by transferring sub-samples from the reacting tube into fibrinogen solution. The first appearance of thrombin was delayed until 11 minutes; it never reached normal concentration, and its disappearance was also delayed; on occasion some was still present at the end of an hour. The range of sensitivity of the patient's plasma to variations in the calcium concentration was normal, as was its sensitivity to added thrombin.

The Absence of Inhibitors.—The addition of one volume of the patient's plasma to one volume of normal plasma caused no increase in the recalcification clotting time when compared with that of normal plasma diluted with one volume of saline. Tocantins, Carroll, and Holburn (1951) have shown that by diluting normal plasma with saline the recalcification time is first shortened, and in some patients the presence of an inhibitor may be exposed. The behaviour of this patient's plasma in such an experiment was comparable with that of normal plasma. The sensitivity of the patient's plasma to added thromboplastin, both brain and Russell viper venom, was compared with that of normal plasma. The plasma clotting time was shortened by high dilutions of thromboplastin, suggesting again that no inhibitor was present. The shortening was, however, not quite as great as that of normal plasma, but was of the same order as that found in haemophilic plasmas (Biggs and Macfarlane, 1951).

Activity of the Patient's Platelets.—Platelet suspensions from the patient were added, undiluted and in dilution, to high-spun plasma, and the accelerating effect on the coagulation time of these additions was compared with the coagulation time of the recalcified plasma alone. A normal platelet suspension prepared in parallel was used as a control. The acceleration effect on high-spun plasma from both the patient and a normal person was investigated at the same time.

From the results reported in Table III it will be seen that the suspensions of the patient's platelets are nearly, but not quite, as efficient as the normal control in accelerating the clotting of normal plasma, even when the suspension is considerably diluted. In contrast, the patient's platelets are considerably less efficient than the normal platelets in accelerating the clotting of her own high-spun plasma. It will have been observed that the platelet suspensions were not washed. Contamination with plasma might have explained this dis-

TABLE III

COMPARISON OF PATIENT'S PLATELET ACTIVITY

Dilution of Platelet Suspension		1/1	1/5	1/25	1/125	1/625	1/3,225	Saline
Normal high-spun plasma	Patient's platelets	1·50	1·55	2·55	2·35	2·32	3·0	3·45
	Normal platelets	1·30	1·10	1·30	2·05	3·0	2·45	3·45
Patient's high-spun plasma	Patient's platelets	6·0	7·0	10·15	11·30	12·5	17·0	18·0
	Normal platelets	1·45	4·0	7·10	5·30	8·40	12·15	18·0

Time in minutes and seconds. To 0·2 ml. of the plasma, 0·2 ml. of the platelet dilution was added, and finally 0·2 ml. of M-40 $CaCl_2$; 0·2 ml. of normal saline was added in place of the platelet suspension in the control tubes.

crepancy, but dilutions of normal high-spun plasma greater than could have occurred in the preparation of the platelets had a minimal accelerating effect. These results suggest that the patient's platelets were at fault and that the plasma might lack a substance which normally reacts with platelets to produce thromboplastin.

Antihaemophilic Globulin.—The presence of antihaemophilic globulin in a patient's plasma can be demonstrated if haemophilic plasma corrects the patient's clotting defect as efficiently as does normal plasma, or if conversely a patient's plasma corrects the clotting defect of haemophilic plasma.

Four haemophiliacs were available. They all had typical clinical histories, long clotting times, an impaired prothrombin consumption, and were corrected by the addition of small quantities of normal plasma and were not significantly mutually correcting. (Later it was shown that none was a case of Christmas disease.)

TABLE IV

CLOTTING TIME OF MIXTURES OF PATIENT'S PLASMA AND HAEMOPHILIC PLASMAS AND DILUTIONS OF BOTH

Mixture	P + S	P + H	P/10 + H	P/100 + H	S + H	P + N/10
Nov. 9, 1951 ..	8·20	7·25	9·08	—	14·0	4·32
,, 21, 1951 ..	7·25	5·10	8·50	9·30	15·30	
Dec. 5, 1951 ..	4·40	4·07	6·15	7·30	14·0	2·50

Time in minutes and seconds. P=0·1 ml. of patient's plasma, H=0·1 ml. of haemophilic plasma, H/10, H/100, P/10, P/100=0·1 ml. of 1/10 or 1/100 saline dilution of the appropriate plasma, N/10=1/10 dilution in saline of normal fresh plasma. 0·1 or 0·2 ml. of $CaCl_2$ added as appropriate. S=0·1 ml. 0·85% saline.

The results in Table IV represent the average figures from a number of experiments on each day on each of a number of different haemophiliacs. They have been separated into batches according to the date the experiments were carried out, because during this period the patient's clotting time was rapidly approaching normal.

In no case when equal parts of the patient's plasma and haemophilic blood were mixed was the clotting time appreciably shorter than the clotting time of the patient's plasma alone. These results may be compared with the definite shortening produced by even a tenth part of normal plasma. It will be seen that a tenth part, or even a hundredth part, of the patient's plasma appears to have some slight effect on the clotting of the haemophilic plasmas, but this effect was not nearly so great as that usually obtained with similar dilutions of normal plasma. It must be concluded that the patient's plasma was grossly deficient in antihaemophilic globulin.

It is known that the clotting defect of haemophilic plasma is apparently corrected by the addition of small quantities of normal plasma. The patient's plasma was found to be markedly corrected by the addition of normal plasma in high dilution, but the degree of correction was perhaps not quite as marked as occurs in true haemophilic blood.

Presence of the Christmas Factor.—The investigation of the reaction of the patient's plasma with haemophilic plasma was delayed until she had nearly recovered, because in the first stages of the investigations (in October, 1951) the patient and a haemophiliac were venepunctured simultaneously and the two bloods mixed in equal proportions. The mixture clotted in 6 min. 45 sec., whereas the patient's blood clotted in 31 min. and that of the haemophiliac in 16 min. The prothrombin consumption index—grossly abnormal in the patient and the haemophiliac—was in the mixed blood also corrected almost to normal. The results were amplified by studies of the plasmas. It was only later that this "haemophiliac" was found to be a case of Christmas disease (Biggs, Douglas, Macfarlane, Dacie, Pitney, Merskey, and O'Brien, 1952). The present patient's blood, therefore, does not lack the Christmas factor.

Summary and Conclusions

The literature of acquired coagulation defects in women is briefly reviewed. A patient had large subcutaneous bruises and haematuria three months after her first confinement. She appeared to have recovered completely four months later. Her bleeding time was rather long and her coagulation mechanism defective. No other abnormality was found. The formation of thrombin was delayed and incomplete, and the prothrombin consumption grossly abnormal. The one- and two-stage prothrombin times were normal, and there was

no evidence of an inhibitor. The prothrombin was therefore normal, but the ability to form thromboplastin was abnormal. She appeared to lack antihaemophilic globulin, but not the Christmas factor. The platelets must also be regarded as having been abnormal. They were not nearly as efficient as normal platelets in accelerating the clotting of her own high-spun plasma, but were almost as efficient as normal platelets in accelerating the clotting of normal high-spun plasma. Her recovery prevented the fullest investigation, and it is particularly regretted that the thromboplastin generation test (Biggs and Douglas, 1953) had not then been devised.

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"ACQUIRED HAEMOPHILIA-LIKE DISEASE"

REPORT OF A CASE WITH STUDIES ON THE MODE OF ACTION OF THE CIRCULATING ANTICOAGULANT

BY

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In recent years an increasing number of cases of haemorrhagic diathesis with long clotting times have been shown to be due to a circulating anticoagulant. Nineteen of these cases have recently been reviewed by Collins and Ferriman (1952) and several other examples have been reported. These cases include a group of haemophiliacs, in whose plasma the development of anticoagulant activity has followed the therapeutic administration of blood or blood products, and a group of other patients in whom the anticoagulant has appeared spontaneously, sometimes in association with some other disease or following a pregnancy. "Acquired haemophilia-like disease" would appear to be the most satisfactory name for this second group of cases in the present state of knowledge of their aetiology.

There is good evidence that the anticoagulant of the haemophilic group is an antibody to antihaemophilic globulin produced in response to its repeated therapeutic administration, usually in the form of blood transfusions (Craddock and Lawrence, 1947; Frommeyer, Epstein, and Taylor, 1950). The exact nature and mode of action of the anticoagulants of the second group, on the other hand, remain obscure. It appears from the more fully investigated cases that they interfere with the formation of plasma thromboplastin, but the manner in which they do so remains in doubt.

A further "non-haemophilic" case is now presented in which the anticoagulant appeared to be an inhibitor of antihaemophilic globulin.

Clinical Report

The patient was a 71-year-old unmarried woman who had had rheumatic heart disease since childhood. In May, 1952, she was admitted to another hospital in severe heart failure with auricular fibrillation, and with large bruises on both arms. She gave a history of repeated spontaneous bruising of the arms and legs for the previous five months. The onset of this bruising tendency was apparently unrelated to injury or illness, and she had never had any blood transfusions. There was no family history of any bleeding disorder, and at no time had she had purpura, haemarthrosis, or haemorrhage from mucosal surfaces, with the exception of one episode of haematuria about three years previously.

Blood examination on May 5, 1952, gave the following results: Hb, 6.8 g. per 100 ml.; R.B.C.s, 2,620,000; W.B.C.s, 14,400 (polymorphs 70%, lymphocytes 28%, monocytes 2%); reticulocytes, 8.8% of R.B.C.s; bleeding time (Duke), 2 minutes; clotting time (Lee and White), 25 minutes; prothrombin time (Quick), 17 seconds (control 15 seconds); platelets, 214,000 per c.mm. A sternal marrow puncture on May 26 revealed a cellular marrow showing normoblastic hyperplasia.

The patient was treated with digoxin, "neptal," and blood transfusions, and was discharged on June 13. Three days after discharge she began to develop fresh bruises and she was readmitted to hospital on August 11 with Hb 7.1 g. per 100 ml. and a clotting time of 70 minutes. She was again treated with blood transfusions, but continued to develop spontaneous haematomata of varying size. On September 20 she began to complain of abdominal pain, and two days later she was transferred to St. Thomas's Hospital under the care of Dr. J. S. Richardson. On admission she was found to have a number of large bruises and deep, subcutaneous haematomata at various stages. All these, she stated, had arisen spontaneously without injury. During the next few days she developed generalized abdominal pain with increasing abdominal distension and tenderness, and began to vomit. On September 26 bowel sounds became practically inaudible, and on the following day she became shocked and cyanosed and died, five days after admission to St. Thomas's Hospital.

Necropsy revealed an area of haemorrhage into the wall of the terminal part of the ileum which appeared to have caused a venous infarction. There was a haemoperitoneum and haemorrhage into both lungs and the posterior mediastinum. The heart was of normal size and showed slight stenosis of the mitral valve. Death was evidently chiefly due to the intestinal haemorrhage.

Laboratory Investigations

General Methods.—All blood used in coagulation studies was collected by clean venepuncture: plasma was obtained by mixing nine parts of venous blood with one part of 3.1% sodium citrate, and centrifuging at 2,500 r.p.m. for 10 minutes. The source of brain thromboplastin was a buffered saline extract of human brain prepared by the method of Owren (1949), stored at −20° until required, and thawed at 37° immediately before use.

Routine Investigations.—The results of the routine laboratory investigations carried out on admission to St. Thomas's Hospital were as follows:

Hb, 12.7 g. per 100 ml.; platelets, 312,000 per c.mm.; platelet morphology, normal; bleeding time (Duke), 2 minutes; clotting time (Lee and White), 34–35 minutes; prothrombin time (Quick), 14 seconds (control 14 seconds); prothrombin consumption index (Merskey), 104% (normal < 40%).

Demonstration and Mode of Action of a Circulating Anticoagulant.—Table I shows the clotting times of mixtures of various proportions of normal, haemophilic, and the patient's plasmas.

TABLE I

DEMONSTRATION OF AN ANTICOAGULANT IN THE PATIENT'S PLASMA*

Undiluted Plasma	Diluted Plasma	Plasma Dilutions (%)							Saline
		100	50	30	15	6	3	1·5	0·75	—
		Clotting Times (Minutes)								
Normal ..	Patient	5¼	5	2¾	2¾	2¾	2¾	2¾	2	2¾
Normal ..	Haemophilic	2	2	2	2¾	2¾	2¾	2¾	2¾	2¾
Patient ..	Normal	5¼	5¼	6¼	6¾	6¾	9¼	11	10	12
Haemophilic	Normal	—	—	2¾	2¾	2¾	3	—	3¼	8¾
Haemophilic	Patient	—	—	12	9¼	—	9¾	8¼	9¾	8¾
Patient ..	Haemophilic	—	—	12¾	11¼	12	13¼	11¼	13	12

*0·2 ml. undiluted plasma, 0·2 ml. diluted plasma (or normal saline), 0·2 ml. 25 mM $CaCl_2$.

These results show that the patient's plasma contained an anticoagulant, a 50% dilution of which was capable of prolonging the clotting time of normal plasma. Although the patient's plasma did not correct the haemophilic defect, its effect on normal plasma served to exclude the diagnosis of haemophilia. A further difference from haemophilia is shown by the failure of high dilutions of normal plasma to correct the patient's defect.

Heparin-like Substances.—An excess of such substances was excluded by titration of the patient's plasma with toluidine blue.

Antithrombin.—An antithrombin titration, by a modification of the method of Quick (1938), showed a lower titre in the patient's plasma than in a normal control.

Inhibitors of Human Brain Thromboplastin.—The reaction of the patient's plasma with serial dilutions of human brain thromboplastin was comparable with that of haemophilic plasma, and provided no evidence of the presence of an inhibitor of the thromboplastin.

Inhibitors of Blood Thromboplastin.—The patient's plasma clotted in the same time as a normal control plasma on the addition of blood thromboplastin prepared by the method of Biggs, Douglas, and Macfarlane (1953).

Inhibitors of Blood Thromboplastin Generation.—The results so far reported are compatible with the presence in the patient's plasma of an inhibitor of blood thromboplastin generation. Such a substance might act as an antagonist of either the platelet factor or of one of the plasma factors concerned in this process, or conceivably by preventing the interaction of two or more of these principles. The following two investigations suggest that the anticoagulant in this case was an inhibitor of antihaemophilic globulin.

First, Table II shows the effect of normal plasma and the patient's plasma, separately and in combination, on the clotting time of haemophilic blood.

TABLE II

NEUTRALIZATION OF ANTIHAEMOPHILIC GLOBULIN BY THE PATIENT'S PLASMA

Haemophilic whole blood (ml.)	1·0	1·0	1·0	1·0
Normal plasma (ml.)	—	0·1	—	0·1
Patient's plasma (ml.)	—	—	0·1	0·1
Citrate-saline (ml.)	—	0·1	0·1	—
25 mM $CaCl_2$ (ml.)	—	0·2	0·2	0·2
Clotting time (minutes) ..	21	3 3¼ 3¼	19 19¼ 20	8¼ 9 10¼

It is apparent from Table II that the patient's plasma not only failed to shorten the clotting time of haemophilic blood, but partially prevented the normal plasma from doing so. It may reasonably be inferred that this effect was due to a direct action on the antihaemophilic globulin of the normal plasma. The small proportion of normal plasma used, relative to the volume of haemophilic blood, virtually excludes the possibility that its correcting effect could have been due to any factor other than antihaemophilic globulin.

Second, it was considered that the anticoagulant in the plasma might take part in a time-consuming reaction with antihaemophilic globulin resulting in the inhibition of the latter substance, and the following experiment was devised to test this hypothesis.

Equal amounts of patient's and normal plasmas were incubated together and 0.1-ml. samples of this mixture were transferred at intervals to 0.1 ml. of haemophilic plasma, 0.2 ml. $CaCl_2$ being added simultaneously. The results are expressed in Fig. 1, which shows that the corrected clotting time of the haemophilic plasma increased progressively with the time of incubation of the normal and patient's plasmas. The initial reading of 120 seconds was obtained by the use of citrate-saline in place of the patient's plasma, and this figure remained constant, irrespective of the length of the first stage. The uncorrected clotting time of the haemophilic plasma was 600 seconds. These results show that the patient's plasma causes a rapid and progressive reduction of the ability of normal plasma to correct the haemophilic defect,

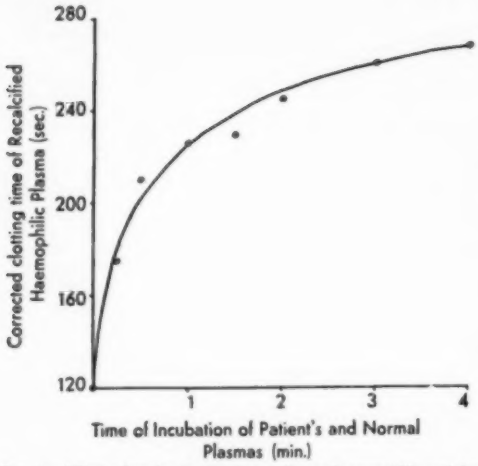

FIG. 1.—Effect of patient's plasma on the antihaemophilic activity of normal plasma.

and one may infer that this was due to a progressive neutralization of antihaemophilic globulin.

Precipitin Tests.—No precipitins could be demonstrated against whole normal plasma or against either of two purified preparations of antihaemophilic globulin.

Physical Properties of the Anticoagulant.—The anticoagulant activity of the patient's plasma remained undiminished after storage at −20° for 19 days, but was completely lost on storage at 37° for three days; the serum was found to possess anticoagulant activity after storage at −20° for six months.

Dialysis of the plasma was not performed, as the quantity available was limited by the patient's early death.

The electrophoretic pattern of the patient's plasma was within normal limits.

The results of a crude ammonium sulphate fractionation experiment suggested that the anticoagulant was contained in the fraction of the patient's plasma precipitated by 33% saturation with ammonium sulphate, and that it acted by the inhibition of some factor which was contained in the same fraction of normal plasma.

Discussion

The finding of a normal one-stage prothrombin time in a patient with a prolonged whole-blood clotting time serves to rule out deficiencies of prothrombin, factors V and VII, and fibrinogen, and to suggest that the defect is one of thromboplastin generation. Such a defect may be brought about by a simple deficiency of one of the other active principles concerned in this stage of the coagulation process—namely platelets, antihaemophilic globulin, or Christmas factor—or by the presence of an inhibitor of one or other of these.

In the present case, haemophilia and Christmas disease could be excluded on clinical grounds alone, as the bleeding disorder started late in life in a woman with a negative family history. The clinical features, platelet count and morphology, and the bleeding time all combined to exclude platelet disorders. The final diagnosis could thus be arrived at by a process of exclusion based on routine clinical and laboratory observations, and was confirmed by further investigations.

That precipitins to antihaemophilic globulin could not be demonstrated in this case does not, of course, rule out the possibility that the inhibitor was a true antibody. That such investigations have given positive results in only a proportion of the haemophilic group is most probably due to technical causes, and these may also account for the failure in this case. Collins and Ferriman (1952) have pointed out that most of the previously described patients in the non-haemophilic group suffered from conditions which may be associated with disturbances of immunity mechanisms, and the patient presented here is no exception to this rule, suffering as she did from chronic rheumatic heart disease. It may be that this condition represents an instance of auto-immunization, analogous to the acquired haemolytic anaemias and thrombocytopenias. Whether all cases of "acquired haemophilia-like disease" are due to inhibitors of antihaemophilic globulin, or whether similar inhibitors of other clotting factors may be responsible for a proportion of them, is a further point which remains to be investigated.

Summary

A case is presented of a haemorrhagic diathesis occurring in a woman of 71 with chronic rheumatic heart disease, and resulting in a fatal intestinal haemorrhage.

Laboratory studies revealed that the condition was due to the presence in the patient's plasma of a circulating anticoagulant.

Evidence is presented that this anticoagulant was an inhibitor of antihaemophilic globulin.

I am indebted to Miss A. York for technical assistance.

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HAEMORRHAGIC DIATHESIS DUE TO DEFICIENCY OF FACTOR VII

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Of recent years it has become apparent that Quick's one-stage prothrombin time, although a most useful routine laboratory test, does not specifically measure plasma prothrombin.

Quick himself (1947) recognized that deficiency of his labile factor (Factor V) caused lengthening of the prothrombin time, and Owren (1947) reported the first case of congenital deficiency of Factor V causing a haemorrhagic state which he termed "parahaemophilia."

It is now known that a prolonged prothrombin time may result from lack of another factor which normally develops in serum during coagulation, and has been named by various workers "serum prothrombin conversion accelerator (Alexander), "convertin" (Owren), and Factor VII (Koller, Loeliger, and Duckert, 1951).

Only two undoubted cases of congenital deficiency of this factor have so far been described, the first by Alexander, Goldstein, Landwehr, and Cook (1951), and another by Owren (1952). The following case appears to be the third example.

Case Report

Mr. R.H., aged 49, was admitted to hospital in November, 1952, complaining of blood in the stools. At the age of 5 he had suffered from epistaxes and haemarthroses of the knee joints. Further epistaxes occurred during school life, and slight trauma caused haemarthroses of the ankle joints at the age of 22. Haemorrhages, mostly epistaxes, continued to occur and in 1946 he began to notice occasional blood in the stools. In April, 1952, rectal bleeding recurred and continued intermittently until admission to hospital. Family history revealed no definite evidence of haemorrhagic disease in his immediate relations.

On admission he was pale with a temperature of 99° F., pulse 90. There was full movement of all joints. The stools contained bright red blood. On investigation Hb was 48% (7.1 g. per 100 ml.), red cells 4.05 million and white cells 6,400 per c.mm., with a normal differential count. The blood sedimentation rate was 3 mm. per hour, the platelets 521,000 per c.mm., bleeding time (Duke) 4 min., and plasma fibrinogen 250 mg. per 100 ml. A barium enema was normal on two occasions, and proctoscopy and sigmoidoscopy revealed no local lesion. Vitamin K in the form of "synkavit" was given in the dose of 100 mg. daily for 17 days by intramuscular injection without effect. Blood transfusions raised the haemoglobin to 94%, and the bleeding gradually declined in severity. He remained well with a haemoglobin value of 92% when seen six weeks after leaving hospital.

Investigation of the Coagulation Defect

Methods.—One-stage prothrombin times were carried out by Quick's method using "difco" rabbit brain thromboplastin. Two-stage prothrombin estimations were carried out by Ware and Seegers' method (1949), using "difco" two-stage reagent, except that Factor V was supplied by $Ca_3(PO_4)_2$-treated plasma and Factor VII by prothrombin-free normal serum both in 1 in 150 dilution.

Prothrombin consumption was determined by the one-stage method of Stefanini and Crosby (1950) modified by the addition of Factor VII when estimating the plasma and serum prothrombin times. Factor V was investigated as shown below.

Plasma containing Factor V, but free from prothrombin and Factor VII, was obtained by treatment with an equal quantity of 0.008 M-tricalcium phosphate suspension, $Ca_3(PO_4)_2$.

The adsorbed prothrombin and Factor VII was obtained in solution by eluting the precipitate with 0.2 M-sodium citrate in a volume one-tenth of the original plasma.

Serum was rendered free from Factor VII by similar treatment with $Ca_3(PO_4)_2$ and the adsorbed Factor VII was obtained in solution by elution of the precipitate with sodium citrate in a volume one-tenth of the original serum.

Coagulation Time.—Determined by the Lee and White method this was five to eight minutes (normal five to ten minutes).

One-stage Prothrombin Times.—These ranged from 39 secs. to 70 secs. (normal 15–17 secs).

Two-stage Prothrombin Test.—Prothrombin by this method was 89% of normal, the patient's plasma containing 144 units and the normal 165 units after two minutes' incubation with supplementary Factor V and Factor VII. If Factor VII was omitted, the value for normal plasma remained the same but the patient's plasma showed a significant delay in thrombin formation and the peak yield was reduced to 127 units after seven minutes' incubation.

Factor V.—Factor V (labile factor) was shown to be normal by two methods: (1) Stored normal plasma gave a prothrombin time of 40 secs. (normal 17) and stored normal plasma plus the patient's plasma in equal parts gave a prothrombin time of 16 secs.; (2) 90% of normal $Ca_3(PO_4)_2$ plasma plus 10% of normal prothrombin plus Factor VII gave a prothrombin time 16 secs., 90% of patient's $Ca_3(PO_4)_2$ plasma plus 10% of normal prothrombin plus Factor VII gave 16 secs., and 90% of normal $Ca_3(PO_4)_2$ plasma plus 10% of patient's prothrombin plus Factor VII gave 42 secs.

This experiment demonstrates that $Ca_3(PO_4)_2$ removes Factor VII from normal plasma but leaves Factor V intact.

Prothrombin Consumption Index.—This, after the addition of Factor VII, was 6% (normal 1%–15%).

Factor VII.—Table I shows the effect of normal serum, normal plasma, and patient's serum on the prothrombin time when mixed in various proportions with the patient's plasma. Ten per cent. of normal serum completely corrected the prothrombin time and was approximately twice as effective as normal plasma. The patient's serum was relatively ineffective.

TABLE I

PLASMA MIXTURES (PARTS)

Patient's Plasma	Patient's Serum	Normal Plasma	Normal Serum	Prothrombin Time (Sec.)
1				50
9			1	17
9		1		23
9	1			35
19			1	22
19	1			42

Prothrombin times were carried out on 0·1 ml. of mixture in each case. Prothrombin time of normal control plasma, 17 secs.

TABLE II

Patient's Plasma	Patient's Pro-thrombin Solution	Factor VII from Normal Serum	Factor VII from Patient's Serum	Normal $Ca_3(PO_4)_2$ Serum	Normal $Ca_3(PO_4)_2$ Plasma	Pro-thrombin Time (Sec.)
1						50
1				1		70
9		1				12
	1				9	60
	1	1			8	13
	1		1		8	80

Prothrombin times were carried out on 0·1 ml. of mixture in each case. Prothrombin time of normal control plasma, 17 secs.

Table II shows the effect of preparations of Factor VII from normal serum and the patient's serum concentrated in a volume one-tenth of the original serum. These preparations were tested on the patient's plasma and on a solution of the patient's prothrombin concentrated in a volume one-tenth of the original plasma.

Again, normal Factor VII was very potent in accelerating the prothrombin time whereas the patient's Factor VII was deficient.

Normal serum treated with $Ca_3(PO_4)_2$ was devoid of Factor VII activity.

"Tromexan" Plasma.—The plasma from a case of coronary thrombosis receiving "tromexan" had little effect: 90% of patient's plasma plus 10% of "tromexan" plasma gave a prothrombin time of 43 secs. and 90% of patient's plasma plus 10% of normal plasma gave a prothrombin time of 22 secs. (The prothrombin time of the patient's plasma was 55 secs., of "tromexan" plasma 33 secs., and of the normal plasma 16 secs.).

Blood Transfusion.—A slight transitory effect was observed: November 25, prothrombin time 46 secs., and 24 hours after 2½ pints stored blood, 36 secs.; December 2, prothrombin time 50 secs., and 12 hours after 3 pints fresh blood, 33 secs.; December 9, prothrombin time 50 secs., during transfusion of fresh blood (after 1½ pints) 28 secs., and 18 hours after the transfusion, 50 secs.

Discussion

Factor VII (Koller *et al.*, 1951) develops in normal serum during coagulation from a precursor in the plasma, and appears to be identical with the "serum prothrombin conversion accelerator" of Alexander and the "convertin" of Owren. Unlike Factor V, it is adsorbed by inorganic precipitates and can be eluted by sodium citrate. It is considerably reduced by administration of "tromexan" or "dicoumarol," and, in fact, the lengthened "prothrombin time" in the first few days of treatment with these drugs is largely due to reduction of Factor VII (Koller *et al.*, 1952). The haemorrhagic diathesis of the case here reported appears to be due to Factor VII deficiency, probably congenital in origin, since haemorrhages had occurred from early childhood. Plasma prothrombin was shown to be normal by the two-stage method in spite of a grossly increased "prothrombin" time. This could be completely corrected *in vitro* by the addition of normal serum, and to a less extent by normal plasma. The serum factor had the properties of Factor VII and the patient's serum was shown to be deficient in this respect. "Tromexan" plasma, being deficient in Factor VII, also had little effect.

The effect of blood transfusions, which were given mainly to correct the anaemia, had a slight

but transitory effect on the prothrombin time. From the *in vitro* experiments the transfusion of normal serum would seem to have the greater promise of correcting the coagulation defect, but in the case of Alexander *et al.* (1951), where this was done, the effect was again of only short duration. Although only two previous cases of this condition have been recorded, it seems probable that some cases of so-called "idiopathic hypoprothrombinaemia" where only one-stage prothrombin times were performed, were examples of Factor VII deficiency.

Recently, Biggs, Douglas, Macfarlane, Dacie, Pitney, Merskey, and O'Brien (1952) have reported a series of cases of a haemorrhagic state closely resembling haemophilia, which they have termed "Christmas disease," due to deficiency of a serum factor having many of the properties of Factor VII. This disease, however, is distinguishable from Factor VII deficiency by a normal prothrombin time and impaired prothrombin consumption.

Summary

A case of a haemorrhagic diathesis present from early childhood is reported.

The prothrombin level was normal in spite of a greatly prolonged Quick's prothrombin time.

The coagulation defect could be completely corrected *in vitro* by serum containing Factor VII.

Treatment by blood transfusion and serum transfusion is discussed.

I wish to thank Dr. R. Bodley Scott for permission to publish this report.

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A MICRO-HAEMATOCRIT FOR DETERMINING THE PACKED CELL VOLUME AND HAEMOGLOBIN CONCENTRATION ON CAPILLARY BLOOD

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The micro-haematocrit to be described is simple to make, easy to use, and highly accurate.

In the past various types of micro-haematocrit tube have been described, roughly divisible into those sealed at one end (Epstein, 1916; Ponder and Saslow, 1930; Mason, 1934; Smith, 1936; Grüneberg, 1942; Meyerstein, 1942; Smith, 1944; Harris, Gilding, and Smart, 1951; Sabine and Nickolai, 1952) and those open at both ends (Gram and Norgaard, 1923; Van Allen, 1925a and b; Guest, 1938; Kato, 1938 and 1940; Miller, 1939; Hamre, 1940; Benditt, Straube, and Humphreys, 1946; Shils, Sass, and Goldwater, 1952).

All these tubes suffer from drawbacks either in design or method of use. A simpler capillary micro-haematocrit tube, open at both ends, heparinized and dried before use, and effectively sealed for spinning by a simple rubber band, was introduced by Rosahn (1931). The present tube is an improved development along the same lines, and, from a single sample of capillary blood, gives both the packed cell volume (P.C.V.) and the haemoglobin (Hb) value.

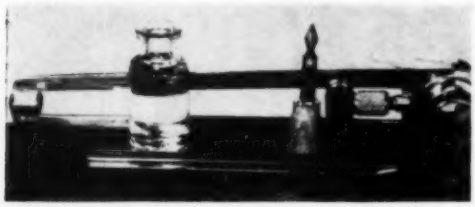

FIG. 1.—Apparatus used: the centrifuge arm (haematocrit head), the micro-haematocrit tube and the stencil cutter, stored in spirit when not in use and fixed for convenience in a cork.

The micro-haematocrit (Fig. 1) is made from "veridia" precision-bore glass tubing* having a guaranteed internal diameter of 0.8 mm. ± 0.01 mm. A length of 10 cm. thus has a volume of 50.3 c.mm. (0.0503 ml.). The tubing was cut to fit our centrifuge arm (Fig. 1), about 11.3 cm. long and bevelled at one end, then marked from this end in millimetres and centimetres up to a length of 10 cm.† Mercury graduation was not used. Alternatively, but less conveniently, the tube can be marked only at 5 and 10 cm., and the P.C.V. measured by a separate millimetre rule.

Technical Procedure

Aqueous heparin solution (1,000 units/ml.) is drawn into the pipette to just beyond the 10-cm. mark, blown out, and the tube then dried in the 56° C. oven.

The pipette, fitted with a rubber mouth-suction tube, is filled with capillary blood from the finger thus: (1) Blood is drawn up to a point about 5 mm. above the 10-cm. mark. (2) The tube is wiped clean, and the upper end of the blood column is brought down exactly to the 10-cm. mark by touching the lower end against the operator's finger. (3) The measured column of blood is next carefully sucked a short distance backwards along the tube and, by means of the suction tube held in the mouth, is made to move backwards and forwards for 30 to 60 seconds in order to heparinize the blood thoroughly. This technique can easily be acquired. (4) After agitation, the column of blood is left in the capillary tube so that a small air space (about 5 mm. long) remains at the bevelled end (Fig. 2). This small air space is important. If the tube is filled at the bedside and has to be transported to the laboratory it is now sealed by a tightly fitting rubber band.

The tube is carefully held in an approximately horizontal position and the rubber band is removed. (The air space at the bevelled end must be retained.) The tube is then inserted into a centrifuge arm of a standard pattern. The point of the tube is pressed against a firm rubber plate in the peripheral end, and screwed in firmly. Alternatively the tube may be screwed into a metal holder of design similar to the centrifuge arm (Fig. 2), or into a reinforced rubber band (Fig. 2). If the latter is to be used for spinning it can be applied in the ward as described above.

* Obtainable from Messrs. Chance Brothers Ltd., Glass Works, Smethwick, 40, Birmingham.

† The length markings were engraved for us by Messrs. Baird & Tatlock (London) Ltd., Freshwater Road, Chadwell Heath, Essex.

The tube, fitted in the centrifuge arm and suitably balanced, or fitted in the metal holder or the reinforced rubber band and simply inserted in the ordinary centrifuge bucket and suitably balanced, is spun at 3,000 r.p.m. for 30 minutes. The P.C.V., as a percentage, can then be read directly ; during the centrifuging the air space is displaced and the top of the blood column comes to lie at the 10-cm. (100%) mark. The reading should be made in a good light and a hand lens used if necessary to define the interface between the buffy layer and the column of packed red cells.

Haemoglobin Estimation.—The method used (Arnold, 1949) is one in which haemoglobin is determined as oxyhaemoglobin. After noting the P.C.V. the bevelled end of the haematocrit tube is immersed in 7 ml. of 0.007N ammonia in a glass tube. After about a minute, to allow separation of the packed erythrocytes plugging the end of the tube, the blood column (0.05 ml.) is blown down into the ammonia solution, which is then transferred to the colorimeter tube and the reading taken.

Results Using Capillary Haematocrit

The P.C.V. and Hb values were determined with the micro-haematocrit as above on samples of venepuncture blood from 50 patients. At the same time these values were determined by routine methods on the same samples of venous blood (Hb determination as above and P.C.V. by Wintrobe's method). A statistical analysis of the results showed the two methods to be interchangeable.

The P.C.V. and Hb values were also determined on single samples of capillary blood from 56 patients, and at the same time these values were determined by routine methods on venepuncture blood from the same 56 patients.

In six cases (Table III) difficulty was experienced in filling the pipette. Statistical analysis of the results in the 50 remaining cases (Table I) showed no significant difference between the mean values of the P.C.V. and Hb as obtained by the two methods. The statistical values indicate that a difference of greater than about 2% for the P.C.V., and about 4% for the Hb, as obtained by the two methods, is unlikely, and a difference of greater than about 3% for the P.C.V. and about 6% for the Hb would be extremely rare.

A similar series of estimations was performed by an independent observer on 18 patients, and statistical ana'ysis again showed no significant difference.

Micro-haematocrit Constants

It can be seen from Table II that the constants for the micro-haematocrit tube compare favourably with those obtained by corresponding routine methods and with those reported by other workers.

Technical Considerations

The Capillary Blood Sample.—The necessity of obtaining blood from the finger without alteration in concentration due to artefact is shown by the results in Table III, in which haemoconcentration (Cases 4, 7, and 28) or haemodilution produced by faulty technique (Cases 1, 23, and 52) is considered to have been a factor.

A free flow of blood is essential, and a stab by a small stencil cutter (Fig. 1) was found to provide a freer flow of blood than is obtained by the

TABLE I

COMPARISON OF VALUES OBTAINED ON CAPILLARY BLOOD BY MICRO-HAEMATOCRIT TUBE AND ON VENOUS BLOOD BY ROUTINE METHODS

Case No.	Micro-haematocrit and Capillary Blood		Routine Methods and Venous Blood		Case No.	Micro-haematocrit and Capillary Blood		Routine Methods and Venous Blood		Case No.	Micro-haematocrit and Capillary Blood		Routine Methods and Venous Blood	
	P.C.V. (%)	Hb* (%)	P.C.V.† (%)	Hb* (%)		P.C.V. (%)	Hb* (%)	P.C.V.† (%)	Hb* (%)		P.C.V. (%)	Hb* (%)	P.C.V.† (%)	Hb* (%)
2	43·5	89	43·5	88	21	47·0	98	47·0	97	40	22·0	43	21·0	42
3	29·0	57	28·5	57	22	41·5	90	43·5	91	41	48·0	104	48·0	104
5	33·0	70	32·0	68	24	26·0	44	26·0	48	42	26·0	51	25·0	49
6	39·0	78	38·0	79	25	23·5	53	25·0	57	43	29·5	54	28·0	50
8	44·5	91	44·5	91	26	36·5	81	36·0	76	44	45·0	99	47·0	103
9	21·0	43	21·0	46	27	25·5	55	26·0	54	45	33·0	69	31·0	66
10	23·5	43	22·0	44	29	48·0	103	45·5	104	46	9·5	23	9·5	23
11	41·0	88	39·0	91	30	19·5	43	20·0	40	47	38·5	80	39·5	83
12	36·5	82	37·0	78	31	20·0	48	19·5	51	48	28·5	62	27·5	62
13	38·5	79	37·5	81	32	40·5	88	41·0	85	49	14·5	34	14·0	33
14	39·0	82	39·0	82	33	35·5	81	37·0	83	50	42·5	94	44·0	93
15	33·0	70	31·0	69	34	46·5	103	47·0	104	51	39·5	88	40·5	86
16	32·5	58	32·5	57	35	23·5	53	25·0	55	53	30·5	65	29·5	64
17	29·5	60	29·0	59	36	21·0	50	20·0	46	54	9·0	20	9·0	19
18	44·5	93	45·0	94	37	34·0	76	34·5	75	55	24·5	48	25·0	48
19	41·5	94	42·0	94	38	21·0	42	22·0	42	56	26·5	50	24·5	46
20	18·5	37	18·0	37	39	27·5	55	26·0	54					

* Hb 100%=16 g. † P.C.V. determined by Wintrobe's method.

TABLE II
MICRO-HAEMATOCRIT CONSTANTS COMPARED WITH THOSE FROM OTHER SOURCES

	Investigation		No. of Observations	Mean ($\bar{x}$.)	Standard Deviation (σ)	Coefficient of Variation (%)	Range ($M \pm 2\sigma$)
Author's micro-haematocrit	P.C.V.*	A	20	20·6	0·3	1·5	20·0–21·2
		B	20	46·7	0·5	1·0	45·7–47·7
	Haemoglobin* (%)	A	30	34·8	0·8	2·3	33·2–36·4
		B	25	106·9	1·3	1·2	104·3–109·5
		C	30	64·6	1·4	2·2	61·8–67·4
Author's use of routine method	Haemoglobin† (%)	A	30	33·9	0·8	2·4	32·3–35·5
		B	26	105·4	1·8	1·7	101·8–109·0
		C	30	65·0	1·0	1·5	63–67
Biggs and Macmillan 1948)	Haemoglobin‡ (%) (neutral grey photometer)		50	97·0	2·9	3·0	91–103
	P.C.V.‡ (Wintrobe haematocrit)		50	45·5	0·4	1·0	44·7–46·3
	P.C.V.§ (micro-method)		20	45·4	0·9	2·0	43·6–47·2
Rosahn (1931)	P.C.V.‖ (micro-method)		100	—	0·2–0·8 (av. 0·5)	0·6–2·2 (av. 1·2)	—

* Determinations made on heparinized venous blood from patients A, B, and C, using three different micro-haematocrit tubes. † Determinations made on the same sample of venous blood as * above, using seven different routine pipettes. ‡ Five different observers each making 10 readings using 10 pipettes or tubes on a single sample of blood. § No details given. ‖ A series of consecutive readings on 10 rabbits using 10 pipettes for each animal.

triangular Hagedorn needle. The pipette should be filled promptly as soon as sufficient blood has collected at the site of puncture. The use of a pipette still wet with heparin solution led to air bubbles in the blood column, with consequent difficulty and delay in filling the tube.

Anticoagulants.—The tube dried out from heparin solution is infinitely easier to use. The efficacy of the heparin has been doubted by Hamre (1940) on the grounds that, if spinning is not done at once, the cells do not pack properly whether heparin be used wet or dry. To test this view, blood from Cases 27–56 (Tables I and III) was heparinized as described earlier, and then allowed to stand in the capillary tube held vertically for varying periods, up to seven hours, before centrifuging. The cells packed properly, and there has been no other evidence pointing to ineffective heparinization.

TABLE III
RESULTS VITIATED BY HAEMOCONCENTRATION OR HAEMODILUTION

Case No.	Micro-haematocrit and Capillary Blood			Routine Methods and Venous Blood		
	P.C.V. (%)	Hb* (%)	M.C.H.C. (%)	P.C.V.† (%)	Hb (%)	M.C.H.C. (%)
1	26·0	54	33·2	30·5	62	32·5
4	26·5	48	29·0	23·5	40	27·2
7	30·0	54	28·8	24·0	45	30·0
23	32·5	71	35·0	38·0	84	35·4
28	53·0	113	34·1	47·5	100	33·7
52	32·0	59	29·5	35·5	66	29·8

* Hb 100% = 16 g. † P.C.V. determined by Wintrobe's method.

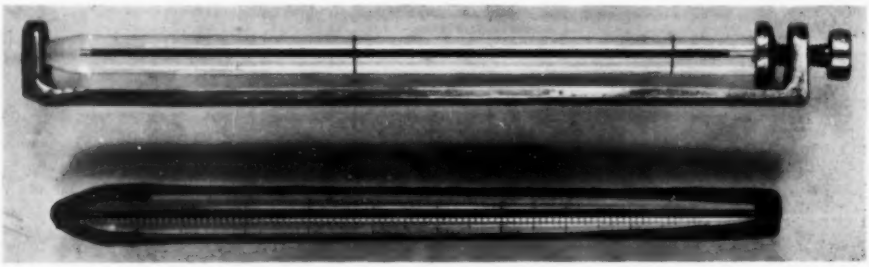

FIG. 2.—Alternative sealing devices: a metal frame, having at one end an adjustable screw and at the other a small firm rubber plate, or a rubber band reinforced by an automobile tyre inner tube rubber patch.

Sealing the Tube.—The variety of methods described in the past for sealing open-ended micro-haematocrit tubes is an index of their unsatisfactoriness. We have found that the standard type of haematocrit head (Fig. 1), or a special metal holder as described by Kato (1938), having at one end an adjustable screw and at the other a small firm rubber plate (Fig. 2), or a rubber band reinforced on its inner surface with an automobile tyre inner tube patch (Fig. 2) ½–¾ of an inch long and fixed by means of a rubber solution, as described by Hamre (1940), serve as perfectly efficient sealing devices. When a reinforced rubber band is used as a sealing device the diameter of the tip of the pipette should be not less than 2.4 mm., the angle of bevel not greater than 70°, and the rubber band should be No. 83 (3 in. long × ½ in. wide). These specifications are important.

The importance of the small air space at the bevelled end is again emphasized, permitting simple insertion of the tube into the sealing device without loss of blood.

The Validity of Absolute Haematological Values Derived from Estimations on Capillary Blood

Haemoconcentration or haemodilution can be produced by manipulations. However, when the P.C.V. and Hb values are derived from the same sample of capillary blood, as is the case with this micro-haematocrit tube, any deviations from the true values for the P.C.V. and Hb, due to concentration or dilution, should be roughly proportional and the mean corpuscular haemoglobin concentration (M.C.H.C.), a value derived from these two determinations, would accordingly be little different from the true value, and this appears to be so, as can be seen in Table III.

The mean corpuscular volume (M.C.V.), on the other hand, is calculated from the P.C.V. and erythrocyte count, estimations made on two separate capillary blood samples. Accordingly, this value is liable to error if any alteration in capillary blood concentration is produced by handling at the time of either sampling. If after the haematocrit has been filled and agitated it is found that after wiping the skin surface with a swab the puncture is no longer bleeding freely, then it is essential to make a fresh stab; otherwise a false erythrocyte count will result if blood is obtained from the original puncture after undue constriction or other gross manipulation of the finger.

According to Wintrobe (1951), "It has been repeatedly shown that there is no difference in counts made on venous blood as compared with capillary blood, if proper precautions to secure a freely flowing sample are observed in each instance."

The haemoglobin content of venous and finger-tip blood shows no significant difference, although blood from the ear lobe contains about 10% more haemoglobin (Brückmann, 1942). A similar observation in cases of pernicious anaemia was reported by Duke and Stofer (1922). Capillary blood from the heel of infants is said to give a higher value than venous blood (Andresen and Mugrage, 1938; Mollison and Cutbush, 1949). These factors, and any degree of shock present, must all be borne in mind when haematological values are being derived from capillary blood.

The Advantages of the Micro-haematocrit Tube

With this simple and yet accurate pipette we are in a position to discover the patient's erythrocyte count, P.C.V., and Hb content without resorting to venepuncture, an obvious advantage in many situations. Also the micro-haematocrit's requirement of only 0.05 ml. may be useful when only a small quantity of blood is available for all investigations. For the experimentalist working with small animals this haematocrit has obvious merits.

Summary

A new micro-haematocrit, made from "veridia" precision-bore glass tubing, is described. It can be filled directly from the finger-tip, and allows determination of both packed cell volume and haemoglobin values on a single sample of capillary blood (0.05 ml.).

This is a simple instrument, and if used as detailed gives extremely accurate results. It is of clinical importance to be able to obtain these haematological values from capillary blood.

I would like to thank Professor A. C. Lendrum for his kind encouragement, assistance, and advice at different stages during the preparation of this paper. I am also deeply indebted to Dr. T. E. Faulkner for the statistical analysis of results. My thanks are also due to Dr. C. G. Woods, who performed the control series of observations, Mr. P. Harrison for the photograph (Fig. 2), and Mr. J. W. Brodie, who made the special metal holder.

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COMBINED AGAR DIFFUSION AND REPLICA PLATING TECHNIQUES IN THE STUDY OF ANTIBACTERIAL SUBSTANCES

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Much interest has recently been aroused in the field of antibiotics by two distinct but related problems. The first concerns the possibility that in certain types of infection, notably subacute bacterial endocarditis, the bactericidal action of a drug is of greater therapeutic significance than the inhibitory effect (Hunter 1950; Robbins and Tompsett, 1951; Cates, Christie, and Garrod, 1951). The second problem is the action of antibiotics used in combination. In this respect the findings of many workers indicate that different mixtures may show augmented or diminished bactericidal effect, and the result may be influenced by the species or strain of organism tested (Jawetz, Gunnison, Bruff, and Coleman 1952; Bliss, Warth, and Long, 1952).

Any investigation of these two problems requires a method applicable to routine use for the assessment of the bactericidal action of antibiotics. None of the conventional methods used for these purposes is suitable for large-scale application, as they depend on some form of viable counting and are very laborious. Even the more convenient turbidimetric technique described by Burnell and Kirby (1951) is too elaborate for general use. Moreover, a disadvantage common to all these methods is that an appreciable amount of drug is inevitably transferred with any sample to the new medium used to measure the bactericidal action. If the proportion of surviving bacteria is low it may be necessary either to limit the range of concentration of the drug under study, or to destroy it in some way which does not affect the bacteria. This is not always possible or convenient. A method of transfer which carries over little more than the amount of drug adhering to individual organisms is therefore needed. The method described here, which is an application of the technique of "replica plating" described by Lederberg and Lederberg (1952) and used by them for studying problems of bacterial genetics, is believed to fulfil these requirements. It involves no significant transfer of drug from one medium to another, is simple enough for routine use, and may be applied as a continuation of a form of "sensitivity test" in common use. It consists essentially of the transfer of bacteria from one nutrient agar surface to another without disturbing their spatial relationships. The transferring agent is the pile of a piece of sterile velvet covering a flat surface. The success of the technique depends on the fact that moisture is absorbed by the pile and no lateral smearing of the impression occurs.

Materials

Construction of the "Stamps."—Discs, 3 cm. thick and of a diameter slightly less than that of the Petri dish with which they are to be used, are made from well-seasoned, close-grained wood. The parallel faces are "band-faced" to give a smooth plane surface. An even layer of "copydex" latex adhesive sufficiently thick to hide the grain of the wood is rapidly brushed over one face and a piece of velvet is smoothed on to it with the pile facing away from the wood. After about two minutes the edge of the velvet is trimmed to the size of the disc. Good-quality furnishing velour of about 2.5 mm. thickness with a close pile has been found suitable: dress material is unsatisfactory. A set of stamps so prepared is sterilized by autoclaving: any which become warped as a result have to be discarded. Pairs of stamps with the velvet faces in contact can be stored ready for use. One stamp may be used many times, but if its velvet surface becomes matted or uneven, the fabric should be replaced.

Primary Plates.—Nutrient media must be firm enough to withstand moderate pressure without splitting: New Zealand agar in 2% concentration has proved suitable. Plates are dried at 37° C. for two hours and the surface inoculated by flooding with young broth cultures just beginning to show turbidity. The inoculum density should be such as to give just

FIG. 1.—The use of the velvet pad in making a replica of a routine "sensitivity plate."

confluent growth. Excess fluid is pipetted off and if plates have been inoculated while still warm from the incubator they are found to dry off within five minutes.

Replica Plates.—These also contain 2% agar and the nutrient base most suited for the development of the particular bacteria under test.

Experimental

Stamping.—The velvet surface of a sterilized stamp is pressed evenly and firmly against the primary plate, avoiding lateral movement. The pad is then lifted off and applied in a similar way to the replica plate (Fig. 1). Alternatively, each plate may be pressed in turn against the upturned surface of a stamp.

Proportion of Organisms Transferred by Replica Plating.—For quantitative interpretation of the results the fraction of the total viable population transferred by the velvet must be known. This was estimated in the following ways:—(1) Serial tenfold dilutions were prepared from a young broth culture and nutrient agar plates were flooded with each. After drying replica transfers were made with small stamps (3 cm. square) which left more than half of the surface of the primary plate undisturbed. Primary and replica plates were incubated and the resulting growths compared (Fig. 2). It is evident that in the range of inoculum employed, approximately one organism out of a hundred has been transferred to the replica plate. For more accurate assessment, the number of colonies in a sample area of 4 sq. cm. was counted on the plates carrying colonies sufficiently dispersed. The experiment was repeated three times with a staphylococcus as test organism and three times with a coliform bacillus. The results showed a transfer of between 1 in 60 and 1 in 27 (average 1 in 40) of the organisms on the primary plate. It was also estimated that the surface inoculation produced by flooding a plate with an undiluted young broth culture was of the order of 5 million bacteria per sq. cm.

(2) The transfer factor of about 1 in 40 obtained by the method described was of necessity determined from primary plates carrying not more than a few hundred bacteria per sq. cm. It seemed possible that a much smaller fraction of organisms might be transferred from an area bearing several millions of bacteria per sq. cm., and it was important to determine this directly, since this was the inoculum density chosen for the subsequent experiments. The method of Burnell and Kirby (1951) was found to be suitable for this purpose. Primary and replica plates of serial tenfold dilutions of a broth culture were prepared in the manner just described. As soon as the plates

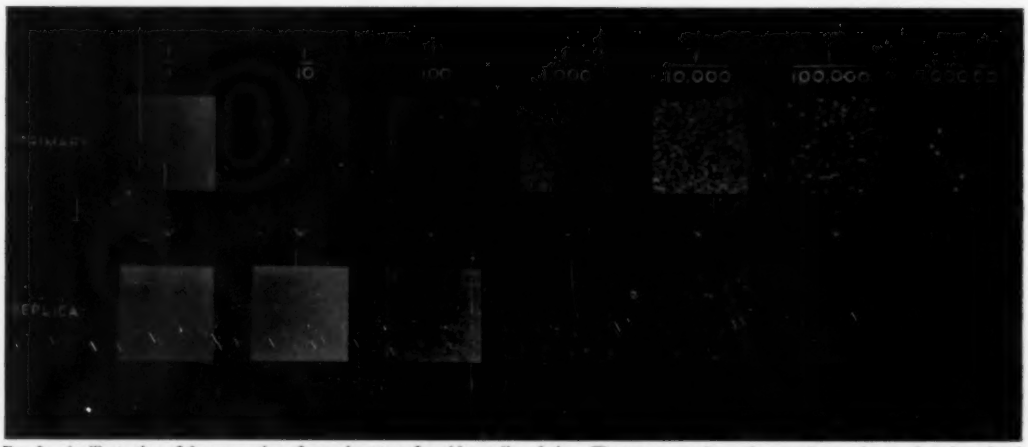

FIG. 2.—An illustration of the proportion of organisms transferred by replica plating. The upper row shows the growth on primary plates inoculated with serial tenfold dilutions of a staphylococcal broth culture. Replica samples were taken from each before incubation, and these are shown in the lower row.

had dried a sample was taken from each plate by punching out a disc of agar with a cork-borer 1 cm. in diameter. Each disc was dropped into a test-tube (150 mm. × 16 mm.) containing 5 ml. of broth; the tubes were shaken vigorously at once and incubated in a water-bath at 37° C. The development of turbidity in the tubes was recorded at 20-min. intervals in an absorptiometer. Turbidity curves were plotted for each tube, the point of 50% maximum turbidity determined for each curve, and a note made of the time taken for this degree of turbidity to be reached. Burnell and Kirby showed that this value is proportional to the number of viable organisms inoculated into a tube. The results of this experiment indicated that the sample disc of agar from a primary plate carried roughly one hundred times as many organisms as did the sample from the corresponding replica plate. This relationship was found with the largest inoculum and its first dilution. There is therefore good agreement between the two methods used in estimating the proportion of organisms transferred by replica plating. It is of the order of 1 in 100 with the density of inocula used.

Estimation of Bactericidal Action

Method 1.—In this procedure predetermined amounts of the drug to be tested are incorporated evenly in the medium and replica samples are taken to record its lethal action. Primary plates are poured with a series of increasing dilutions of the drug and inoculated in the way described. Replica samples are taken from each plate at intervals during incubation using stamps 3 cm. square. As many as 12 samples can be taken from a single large plate (14 cm. in diameter). In this way it is possible to determine the rate at which the different concentrations of drug in each primary plate prove lethal. Although this method is less accurate than a series of viable counts, it may find a useful application in the testing of antiseptics. These are usually used in relatively high concentration and it is notoriously difficult to eliminate errors due to the transference of antiseptics; the replica method is free from this objection.

Method 2.—This procedure is more suitable for routine use with antibiotics. A "sensitivity plate" is prepared according to the paper disc method of Fairbrother and Martyn (1951), using a young broth culture of the organism to be tested. It may be convenient to test the action of up to four antibiotics on one 9 cm. Petri dish. The concentration of antibiotic with which a paper disc is soaked should be about 20 times as great as the maximum concentration which can be achieved in the body by normal therapeutic dosage, since the diffusion of the drug into a layer of agar 4 mm.

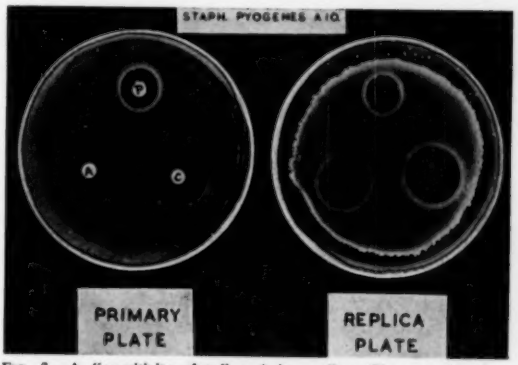

FIG. 3.—A "sensitivity plate" and its replica. The organism is a coagulase-positive staphylococcus. P: penicillin; A: aureomycin; C: chloramphenicol.

deep (the usual depth) produces a dilution of about 1 in 20 in the medium underlying the paper disc. In practice, such concentrations will produce zones of inhibition of a sensitive organism of 15 to 20 mm. diameter after overnight incubation. The zones produced by each antibiotic are measured. A replica plate is made and incubated. An example of a primary plate and its replica is shown in Fig. 3. The lethal action of aureomycin and penicillin on the staphylococcal inoculum has been virtually complete and the diameter of its zone is as large as that of the inhibition zone on the primary plate. The zone of lethal action of chloramphenicol is also equal in size to its inhibitory zone but the bactericidal effect is markedly less than that of the other two antibiotics.

The degree of lethal action may be estimated by comparing the size of the replica zone with that of the inhibition zone and by comparing the colony density within it with a reference chart similar to Fig. 2. The bactericidal effect is recorded as + when no more than a few colonies develop within the zone (approximately 1 in 10,000 survival) and ± for more crowded, discrete colonies. Semi-confluent or greater growth is taken to indicate no significant bactericidal effect. For example, the lethal effect of aureomycin and penicillin in Fig. 3 would be recorded as + while that of chloramphenicol would be ±. Thus a simple visual assessment is afforded of the bactericidal action of a drug on a given strain.

This method has been used to test the action of seven antibiotics against 65 strains of bacteria of different species. The detailed results will be reported later, but certain aspects of interpretation which arose during the investigation must be

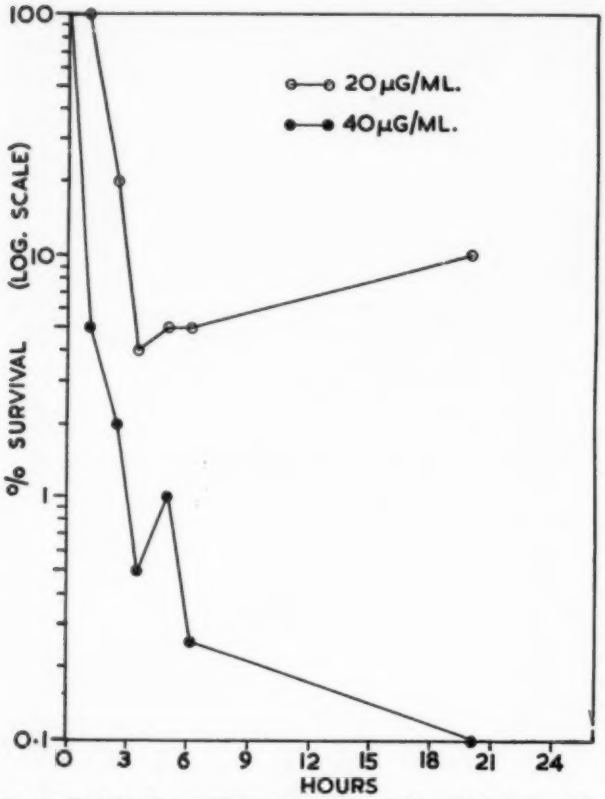

FIG. 4.—The lethal effect of different concentrations of chloramphenicol on a strain of *Bact. coli*.

pointed out. In considering them the dynamic nature of the diffusion system should be borne in mind. Humphrey and Lightbown (1952) have shown that with the concentrations used the amount of antibiotic rises near the central disc during the first hour to a maximum of some 10 or 15 times the minimum inhibitory concentration. It then diminishes steadily as outward diffusion occurs until at 20 hours it has fallen to a value of about four times the minimum inhibitory concentration. Experience has shown that the boundary of the zone of inhibition becomes established after about four to five hours' incubation, and it may be concluded that the minimum inhibitory concentration is attained at this time. The concentration of antibiotic in this area rises slightly further and is then maintained for 20 hours or more. With most drugs, of course, the zone in which the minimum inhibitory concentration is reached continues to expand but its influence passes unnoticed since growth of the organisms has already taken place. If, however, the minimum inhibitory concentration is also rapidly lethal, the organisms lying just outside the boundary of the inhibition zone are killed: this is readily demonstrated by the replica method, the zone of lethal effect being then larger than the zone of inhibition on the primary plate. The expansion of the zone of minimum inhibitory concentration does not always occur. Humphrey and Lightbown (1952) have found that when streptomycin is tested against *Staph. aureus* there is a very sharp decrease in the concentration of the drug just outside the boundary of the inhibition zone. They believe that this is due to the adsorption of streptomycin to these Gram-positive organisms preventing its effective concentration from reaching the minimum inhibitory level beyond the boundary. It is clear that the lethal effect detected by the replica method will not then have a zone size greater than the zone of inhibition. A third possibility is that the size of the zone of lethal effect is considerably smaller than the inhibitory zone on the primary plate. This has in practice been found to occur with many antibiotics and organisms tested. It is interpreted as indicating that a concentration several times higher than the minimum inhibitory concentration is necessary to kill the bacteria within the time during which the primary plate is incubated, and that such a concentration is reached only towards the centre of the area of diffusion. Confirmation for this interpretation is afforded by the results of the following experiment:

A strain of *Bact. coli* and chloramphenicol were chosen because they gave a wide difference between the zone of inhibition on the primary plate (diameter 25 mm.) and the zone of lethal effect on the replica plate (diameter only 13 mm.). The rates of lethal action of different concentrations of the drug were tested by Method 1. The concentrations of chloramphenicol used were 80, 40, 20, 10, 5, and 2.5 μg. per ml. Replica samples were taken at 20-minute intervals for the first hour, then at hourly intervals for five hours, and finally after 20 hours. The minimum inhibitory concentration in this

titration was taken as 10 μg. per ml. since no inhibition occurred with the lower concentrations. The results with 40 μg. and 20 μg. per ml. are shown graphically in Fig. 4. From this it can be seen that twice the minimum inhibitory concentration produced only a 90% reduction of viable organisms after 20 hours, while four times the minimum inhibitory concentration killed 99% within five hours and 99.9% within 20 hours. These findings support the view that if the zone of lethal action is markedly smaller than the inhibition zone, the minimum lethal concentration of the antibiotic for the organism tested is significantly higher than the minimum inhibitory concentration.

Antibiotic Interactions

The demonstration of interactions between antibiotics becomes a relatively simple matter with the technique of agar diffusion combined with replica plating, and can be carried out in a number of ways.

Method 3: "Incorporation and Diffusion System."—This method is an extension of that described in Method 1 above for the estimation of bactericidal effect. A nutrient agar plate is poured with medium containing one drug, usually in a concentration which is just high enough to inhibit the growth of the inoculum. The plate is flooded with a young broth culture and dried rapidly. A blotting paper disc containing the second antibiotic is placed on it without delay. The plate is incubated for any chosen period of time and a replica plate made. The zone in which the organisms have been exposed to the action of both antibiotics is then examined for any difference in the rate of survival compared with the rest of the plate. To give an example, an experiment was performed in which a concentration of chloramphenicol just inhibitory to the staphylococcal inoculum was contained in the primary plate. Blotting paper discs soaked in penicillin, 1, 10, and 100 units per ml., were placed on its surface and the plate incubated overnight. The replica plate made from this is illustrated in Fig. 5, from which it will be seen that over the plate in general there is a moderate degree of survival. However, in the zones where penicillin has also acted on the organisms (which were fully penicillin-sensitive by ordinary testing) the lethal effect of the chloramphenicol is almost completely prevented, showing that there is antagonism between these two antibiotics with this organism. The diameter of the effect is proportional to the original concentration

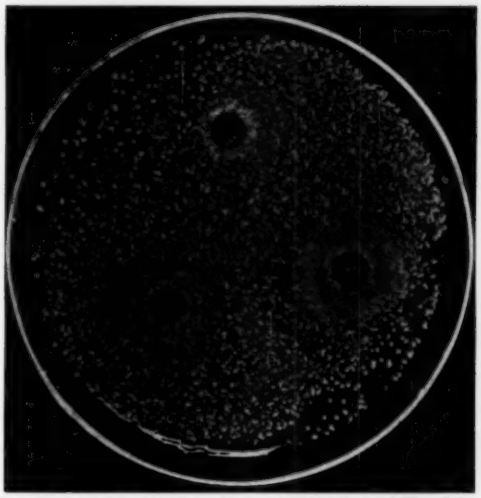

FIG. 5.—A replica plate illustrating antagonism by penicillin of the lethal effect of chloramphenicol on a coagulase-positive staphylococcus. Disc concentrations of penicillin on the primary plate: upper zone, 1 u./ml.; lower left, 10 u./ml.; lower right, 100 u./ml. Chloramphenicol concentration in the primary plate: 15 μg./ml.

of penicillin, and it is clear that the antagonism is not reversed by relatively high concentrations of penicillin.

Method 4: Paper-strips at Right Angles.—This is an application of a technique originally used for serological analysis (Elek, 1949). The principle involved is shown diagrammatically in Fig. 6. When strips saturated with a diffusible substance are placed at right angles to each other on the surface of a plate all combinations of concentrations occur as a result of diffusion in the area

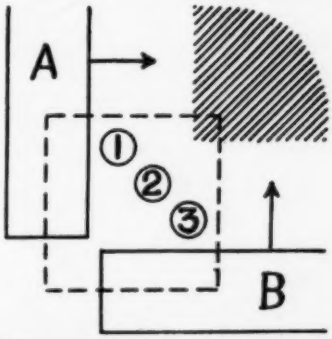

FIG. 6.—Diagram representing the result of diffusion of two antibiotics from paper strips at right angles. At point (1) drug A will be in high concentration, and B in low concentration; at (2) both will be in moderate concentration; at (3) the reverse of (1) exists. The area of shading represents bacterial growth, with inhibition by both drugs.

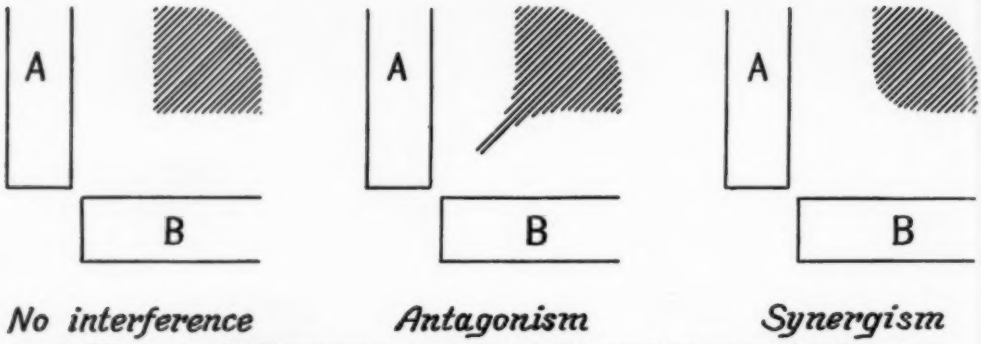

FIG. 7.—Different patterns of inhibition produced on primary plates by the diffusion at right angles of two antibiotics (diagrammatic).

shown. The only limiting factors are the original concentrations in the strips. This method has recently been used for the investigation of inhibitory substances acting on mycobacteria (King, Knox, and Woodroffe, 1953). In practice the experiment is carried out as follows:

A strip of blotting paper, measuring 5 cm. by 0.5–1.0 cm., is moistened evenly by placing it on blotting paper soaked in a solution of one drug. It is placed on the surface of a Petri dish containing nutrient agar previously inoculated with a young broth culture. Another strip containing the second drug is placed at right angles to it to form either a " T " or a cross. The concentrations of the antibiotics used should be approximately 20 times the maximum blood concentration attained with therapeutic dosage. During subsequent incubation bacteria in the angles between the strips will be subjected to different combinations of concentrations of the two drugs. After any chosen period of incubation a note is made of the appearances of the zones of inhibition, the strips are removed and a replica plate is made.

Primary Plates (Fig. 7).—If no interaction demonstrable by bacteriostasis has occurred, the limits of inhibition due to both drugs form a sharp right angle. Antagonism is shown by the presence in the corner of a " tongue " of growth running in towards one of the strips, or, more rarely, towards the angle. This shows that a normally inhibitory concentration of one drug has lost its action in the presence of a sub-inhibitory concentration of the other. Experience with the replica plating method has shown that the appearance of " tongues " correlates with antagonism of lethal action during the first eight hours of incubation of the primary plate; this correlation may be lost during subsequent incubation. With later sampling therefore it may be found that such tongues contain no viable organisms. If there is synergism of inhibition the angle will be rounded off. This indicates that a normally sub-inhibitory concentration of

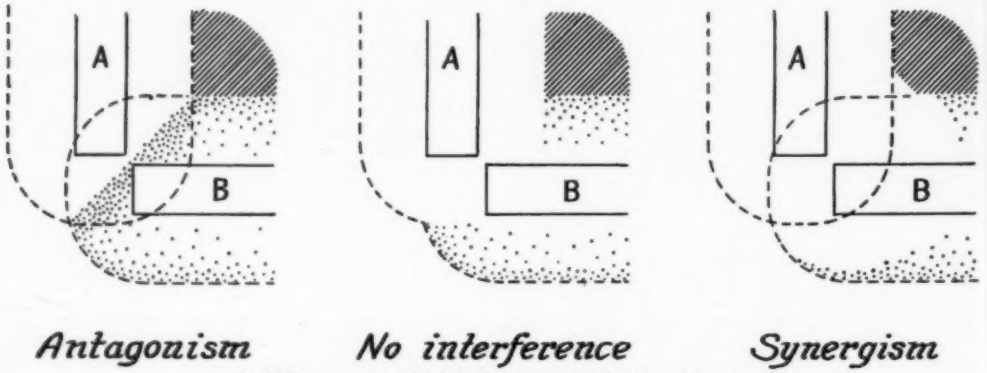

FIG. 8.—Different patterns of lethal effect seen on replica plates (diagrammatic).

one drug has become inhibitory in the presence of a sub-inhibitory concentration of the other.

Replica Plates.—These will reveal the bactericidal action of the combinations. The area nearest to the strip will show the effect of concentrations higher than the minimum inhibitory concentration while the outline or edge of the pattern indicates the effect of combinations near the minimum inhibitory concentration.

The possible interactions between two drugs are shown diagrammatically in Fig. 8. One drug (A) is highly lethal and the other (B) less so. Synergism is revealed by the growth of fewer survivors in the region where the organisms were subjected to the action of both drugs. The angle between the limits of lethal action will also show a rounding off, similar to that described for the primary plate. If both drugs are highly lethal this rounding off may be the only effect demonstrable. Antagonism is indicated by a greater degree of survival in the area of combined action, often accompanying a small tongue of confluent growth extending inwards from the angle between the limits of lethal effect.

All these combinations have been observed with various antibiotics, and photographs illustrating these findings are shown in Fig. 9. In the interpretation of these it must be stressed that the outline indicates the effect on the whole bacterial population, and is the result of antibiotic concentrations close to the minimum inhibitory levels. The central areas show the effects of combinations significantly higher, which are acting on a lesser population—i.e., those organisms that have survived the action just described.

Method 5: Disc Techniques.—Blotting-paper discs may be used to show interactions of antibiotics in two ways: (*a*) by superimposing two discs each containing a different antibiotic on the primary plate, (*b*) by placing the discs side by

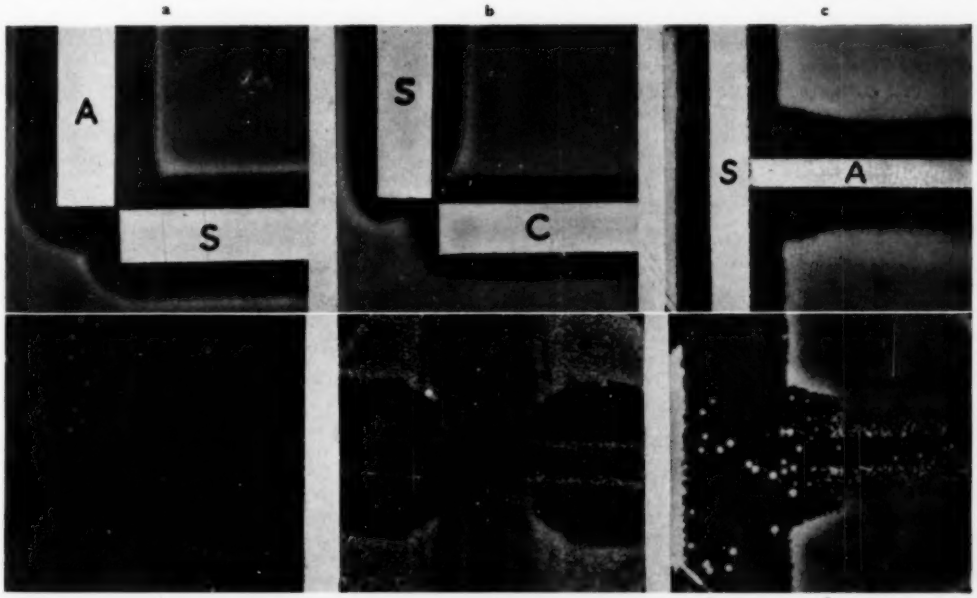

FIG. 9.—Examples of antibiotic interaction: *Primary Plates* (*Inhibition*).—(a) *No interaction* between aureomycin and streptomycin acting on a strain of *Bact. coli*. The inhibition edges form a sharp right angle. (b) *Antagonism* between streptomycin and chloramphenicol acting on a coagulase-positive staphylococcus. There is a "tongue" of growth extending towards the streptomycin strip. (c) *Synergism* between streptomycin and aureomycin acting on a coagulase-positive staphylococcus. The zone of inhibition due to aureomycin is widened in the angle. *Replica Plates* (*Lethal Effect*).—(d) *No interaction* between chloramphenicol (vertical zone) and aureomycin (horizontal) acting on a coagulase-positive staphylococcus. (e) Marked *antagonism* between chloramphenicol (vertical) and penicillin (horizontal) acting on a strain of *Strep. faecalis*. The almost complete lethal effect of the penicillin is lost in the presence of the chloramphenicol, which is itself only feebly lethal. The increased survival close to the strips is probably due to the inoculum being exposed to high concentrations while still in the lag phase, and being prevented from entering the logarithmic phase in which there is greater susceptibility to lethal action. (f) A replica of the plate shown in (c). There is marked *synergism* between streptomycin and aureomycin, shown by the outward extension of the almost complete lethal action of the former where the latter is also present. The synergism is less with the higher concentrations of aureomycin close to the strip.

side, close enough together to produce overlapping of their zones of inhibition. As a control single discs containing each antibiotic are placed on the same plates.

When the discs are superimposed the replica plates show antagonism if the survival with the combination is greater than that with the more lethal antibiotic alone. Synergism is shown by effects similar to those seen with Method 4. On the whole these disc techniques are less satisfactory than the paper-strip methods, since the range of concentrations tested is more limited.

Conclusion

The final judgment of the clinical importance of the lethal action of antibiotics must rest on comparisons between therapeutic results and laboratory findings. Experience has shown that bacterial strains, even of the same species, vary widely in their response to the inhibitory action of antibiotics ; a similar variation is likely in relation to their lethal action. It is felt that the techniques outlined here offer a simple tool which may help towards a solution of these problems.

Summary

Replica plating may be used to provide a simple form of viable count, adequate for practical purposes. Several methods are described based on this principle and designed to reveal the bactericidal action of drugs *in vitro*. The methods may find applications in antiseptic testing, and may be used to assess the lethal action of antibiotics on individual strains isolated from patients and to test for antibiotic interactions.

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THE RAPID ISOLATION OF *MYCOBACTERIUM TUBERCULOSIS* AND THE SIMULTANEOUS STREPTOMYCIN SENSITIVITY DETERMINATION

BY

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One of the difficulties in the rapid laboratory diagnosis of tuberculosis is the time required for a satisfactory growth of the tubercle bacillus to appear on artificial medium. This delay is even more important when it is also desirable to determine the sensitivity of the organism. While in some cases it may not be of paramount importance to obtain a rapid growth of the tubercle bacillus for routine diagnosis, the result of the sensitivity test to be of any great value to the clinician must be available in the shortest possible time. Therefore, the ideal to be aimed at should be the rapid isolation of the organism and the simultaneous determination of its sensitivity.

The medium used for isolating *M. tuberculosis* in many laboratories is that of Lowenstein-Jensen. This medium is satisfactory, but growth of the organism is not usually obtained in less than 14 to 28 days. Slide culture in saponated blood (Giammalvo, Natsios, and Elton, 1949) is more rapid, but the method is too laborious and requires considerable skill. Tarshis and Frisch (1951a and b) and Frisch and Tarshis (1951) demonstrated the value of human blood in culturing the tubercle bacillus.

For sensitivity determinations the serial dilution tube method (M.R.C. Report, 1948) has been widely used but again it is time consuming, and it is necessary to have a culture of the organism already growing. The slide culture method, although accurate, also takes too much time, and is potentially dangerous in a busy laboratory. The incorporation of antibiotic in solid medium is used but requires specially prepared medium and duplicates, both for control purposes and in testing a range of concentrations. The diffusion method of Tinne and Henderson (1950) is convenient but it is essential to keep the bottle upright during the whole period of the test. A more convenient reservoir of the antibiotic is the compressed tablet (Hoyt, 1951), the filter paper strip (Fairbrother and Southall, 1951), or the filter paper disc (Gould and Bowie, 1952).

This paper describes a method whereby the isolation and sensitivity of the tubercle bacillus can be determined simultaneously by adopting the filter paper disc technique and a saponated human blood agar medium. This medium was suggested by Dr. W. M. Levinthal, of the Bacteriology Department, Astley Ainslie Institute, Edinburgh, and, after development by one of us, was found to give more rapid results than the Lowenstein-Jensen medium.

Media

Saponated Blood Agar.—To 95 ml. of citrated group O human blood, 5 ml. of 10% saponin is added, and the mixture heated to 55° for five minutes. Equal quantities of saponated blood and a 4% agar solution (not nutrient) are then mixed at 45°. If kept in the refrigerator this medium will remain suitable for 10 days.

Lowenstein-Jensen Solid Medium (Mackie and McCartney, 1948).—This medium was sloped in 10 ml. amounts in 1-oz. "universal" containers, the containers being held horizontal during solidification. Petri dishes containing 5-mm. thicknesses were also used.

Dubos Fluid Medium (Dubos and Davis, 1946).—This was kept in 1-oz. containers.

Inocula

Cultures.—*M. tuberculosis* H37Rv and other strains were maintained on Lowenstein-Jensen medium and saponated blood agar. The growth was emulsified in the condensation water as evenly as possible, and one standard loopful (gauge wire 2-mm. diameter loop) of this suspension was used as inoculum for each test by spreading evenly over the surface of the medium. Similarly, one standard loopful of Dubos culture was used as an inoculum.

Sputa.—These were all from patients suffering from tuberculosis, and, before use, were examined by Ziehl-

TABLE I

DIFFERENCE IN RATE OF GROWTH OF TUBERCLE BACILLUS ON SAPONATED BLOOD AGAR MEDIUM COMPARED WITH LOWENSTEIN-JENSEN MEDIUM

Inoculum	No. of Specimens Examined	No. of L.J. More Rapid than S.B.A.	No. of Growth Appearing at Same Time	No. where S.B.A. More Rapid than L.J. (Days)											
				1	2	3	4	5	6	7	8	9	10	10+	Total
Sputa ..	115	0	25	1	4	4	11	14	5	5	2	6	6	32	90
Cultures	50	0	15	2	5	2	6	10	1	3	—	—	4	2	35

Neelsen film. Only those specimens which were positive on film examination were accepted. No attempt was made to grade the sputa +, ++, +++, etc. The accepted sputa were concentrated by the acid method. The inoculum was one standard loopful of the concentrate.

Discs

These were prepared from Whatman's No. 1 filter paper (Gould and Bowie, 1952; Bowie and Gould, 1952).

A series of discs of varying strengths was prepared ranging from 0.5 to 100 μg. streptomycin per disc.

Each disc was applied to the centre of the culture area immediately after inoculation. The bottle caps were replaced and Petri dishes sealed with "sellotape." All cultures were incubated at 37° and examined daily for growth and zones of inhibition.

Rate of Growth of M. tuberculosis on Saponated Blood Agar and Lowenstein-Jensen Media

Table I shows that in 90 out of 115 consecutive specimens of sputa the tubercle bacillus grew more rapidly on saponated blood agar than on Lowenstein-Jensen medium. The difference varied from one to more than 10 days. In eight tests the difference was more than 20 days. In five tests no growth was obtained on Lowenstein-Jensen medium, whereas all saponated blood agar cultures produced growth.

The interesting point is that in no test did Lowenstein-Jensen medium produce more rapid growth than saponated blood agar. In 12 of the 25 tests where no difference in the time of appearance of growth was observed, the saponated blood agar medium produced a +++ growth compared with a + on Lowenstein-Jensen.

Fig. 1 illustrates the differences in the time of appearance of growth on the two media.

Table I also shows that out of 50 pure cultures of the tubercle bacillus obtained from sputa in no case did growth appear earlier on Lowenstein-Jensen medium. In 15 instances there was no difference in time between growth on the two media, but even in those the growth was much more abundant on saponated blood agar. In 35 of the 50 pure cultures growth was more rapid on saponated blood agar, the difference varying from one to more than 10 days. Fig. 2 illustrates the difference in the time of appearance of growth on the two media.

Streptomycin Sensitivity Tests on Saponated Blood Agar and Lowenstein-Jensen Media

In these tests the thickness of the medium in the Petri dishes and bottles was approximately uniform.

The zones of inhibition of growth were determined with the H37Rv strain as test organism, and varying strengths of streptomycin per disc. The results are noted in Table II.

Preparation of the Standard Graph

From the results obtained in Table II a graph was drawn by plotting the diameter of the zones of inhibition against the logarithm of the streptomycin concentration per disc. The graph is shown in Fig. 3.

Deduction of Sensitivity from the Standard Graph

With a disc containing an antibiotic there will be a concentration gradient from the disc out-

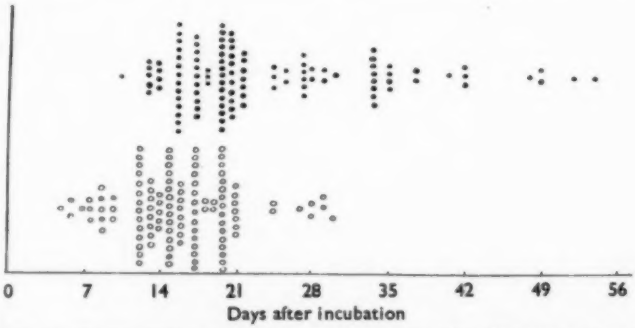

FIG. 1.—Diagram illustrating the times after inoculation when characteristic growth of *M. tuberculosis* first appeared in 115 specimens of sputum. ● Lowenstein-Jensen medium. ○ Saponated blood agar medium.

TABLE II

ZONES OF INHIBITION OF GROWTH OF *M. TUBERCULOSIS* WITH STREPTOMYCIN DISCS OF VARYING STRENGTH

Streptomycin (μg. per Disc)	No. of Tests	Average Diameter Zone of Inhibition on S.B.A. and L.J. (mm.)
0·5	10	No zone
1	20	7
2	21	10
4	23	13
6	13	17
8	15	19
10	105	21
20	22	29
30	16	31
50	16	36
100	16	42

wards, and the zone of inhibition obtained with any test organism will depend on the sensitivity of that organism.

A zone of inhibition of the same diameter as that obtained with the standard organism signifies that the concentration required to inhibit the test organism is the same as that required for the standard organism. If the zone of inhibition with the test organism differs from that obtained with the standard organism the test organism requires a greater or lesser concentration for inhibition than the standard organism.

The sensitivity, i.e., the minimal inhibitory concentration, of any unknown test organism is calculated by reference to the amount of antibiotic per disc required to inhibit the standard organism to the same zone diameter. The sensitivity of our

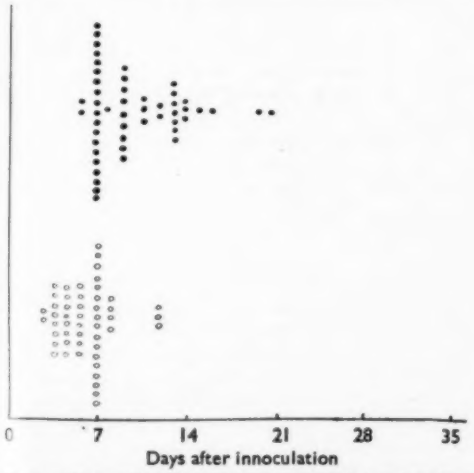

FIG. 2.—Diagram illustrating the times after inoculation when characteristic growth first appeared in subcultures of 50 strains of *M. tuberculosis*. ● Lowenstein-Jensen medium. ○ Saponated blood agar medium.

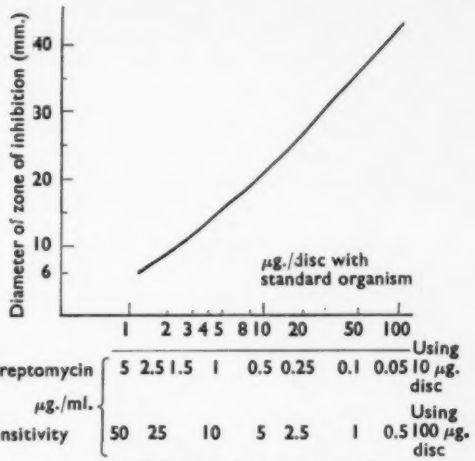

FIG. 3.—Standard graph for streptomycin.

standard *M. tuberculosis* H37Rv is 0.5 μg. per ml. of streptomycin. The sensitivity of the test organism is therefore equal to:

$$\frac{\text{Amount per disc required to inhibit test organism}}{\text{Amount per disc required to inhibit standard organism to the same distance}} \times \text{Sensitivity standard organism}$$

For example: suppose the diameter of the zone of inhibition of a test organism with a standard 10 μg. streptomycin disc is 15 mm., then the graph indicates that a disc containing 4 μg. streptomycin will inhibit the standard organism to 15 mm. Therefore the sensitivity of the test organism is equal to $\frac{10}{4} \times 0.5 = 1.25$ μg. per ml.

Effect of Variable Inoculum.—Sensitivity tests were carried out with the standard strain inoculum varied some tenfold. The results showed that the zones of inhibition of growth did not vary more than 2 mm.

The disc containing 10 μg. of streptomycin, as used for other streptomycin sensitivity tests in this laboratory, was adopted for routine use.

The Standard Test

This may be carried out in one of two ways.

(1) Directly from the sputum: a standard loopful of a specimen of concentrated sputum is inoculated on saponated blood agar or Lowenstein-Jensen slopes as described, and a 10 μg. streptomycin disc applied to the centre of the inoculated area.

The bottles are closed and incubated in the upright position at 37° and examined daily. The zones of inhibition of growth are noted, and reference made to the standard graph.

(2) Pure cultures are used as inoculum and the procedure is as in (1). Sensitive and resistant control strains are also set up with each series of tests.

Time of Reading the Test.—It was noted with several strains of tubercle bacilli tested that the zone of inhibition of growth on saponated blood agar medium tended to become smaller and smaller with continued incubation, whereas on Lowenstein-Jensen medium a zone of inhibition remained.

Discussion

The work reported in this paper demonstrates the advantages of human saponated blood agar as a medium for the isolation of *M. tuberculosis* from material showing the presence of acid-fast bacilli. This medium is easy to prepare and may be stored for an appreciable time in the refrigerator.

The tables show that saponated blood agar medium can yield a quicker growth of the tubercle bacillus than Lowenstein-Jensen medium. It is significant that in no case did Lowenstein-Jensen medium yield a more rapid growth than saponated blood agar; in five tests Lowenstein-Jensen medium failed to show any growth at all, and the saponated blood agar showed growth in every case.

In 36 tests with microscopically positive sputa a growth was seen on saponated blood agar in under 14 days, the shortest time being four days in one case. On Lowenstein-Jensen medium there were only six tests where growth was observed in under 14 days, the shortest time being 11 days in one case. For these reasons it is concluded that saponated human blood agar is a better medium than Lowenstein-Jensen medium for culturing the tubercle bacillus.

The reasons for the saponated blood medium supporting growth of the tubercle bacillus better than Lowenstein-Jensen medium are not known. It may be that there are additional growth factors for the organism present in the blood that are not present in the egg; the saponin, as a surface-active agent, may promote more rapid cell division and so earlier visible growth. Further studies are being carried out on this problem.

Disadvantages of the medium are that the colour of the growth is not so strikingly contrasted with the colour of the medium, but a little experience soon enables one to detect growth in the earliest stages; the medium is not so inhibitory to other organisms likely to be found in sputa and exudates under test so that some care is required in the preparation of inocula direct from pathological material. However, very few cases of contamination were encountered in this series of tests.

The characteristics of the colony of the tubercle bacillus on saponated blood agar are of interest. Early growth is shown by small, circular, convex colonies with an entire edge, bluish-grey and fairly moist. After about three weeks the centre of the colony changes to brown and eventually this change spreads to the periphery. In about six weeks the colony starts to become dry and irregular, but never to such a degree as seen on Lowenstein-Jensen medium. Some colonies after two months exhibit a depressed centre. At this stage, the colony is about 2 mm. in diameter.

Hoyt (1951) and Gould and Bowie (1952) have already shown that the disc diffusion test is adaptable to antibiotic sensitivity determination for *M. tuberculosis*. The slow growth of the organism is no drawback to this type of test since we believe that diffusion outward from the disc is very rapid and, if a bactericidal concentration is reached within a certain radius, it will kill all the cells inoculated on the medium within this area. Thus a zone of inhibition of growth results which is independent of the time of incubation and subsequent diffusion of the antibiotic.

With sputa containing a sufficiently high density of bacilli the standard test with direct inoculum is possible, giving a result at or about the same time as the result of diagnostic culture. This increases the usefulness of sensitivity tests in the treatment of tuberculosis with antibiotic substances. The effect of the quantitative variation in inoculum on the results of such tests is probably of some importance, though not so great as might be expected. Even so, the results are likely to have some value as an indication for therapy, because they may be confirmed by subculture sensitivity tests, which remain the more accurate estimation of *in vitro* sensitivity.

Most of the tests carried out and reported in this series were in the nature of subculture tests. With both Lowenstein-Jensen and saponated blood agar media the striking feature is the reproducibility of the results under reasonably standard conditions. These conditions may be maintained in the routine laboratory, and the reproducibility lends confidence to the interpretation of the results. Thus, with standard discs in use it has been found possible to make the test quantitative, using reference graphs which are detailed in the text, and bringing the sensitivity determination for *M. tuberculosis* into line with the test devised for other bacteria (Gould and Bowie, 1952).

From the graphs it will be seen that with a 10-μg. streptomycin disc a range of sensitivity from 0 to 5 μg./ml. is obtained. This range will include all

those strains of *M. tuberculosis* which are usually described as sensitive to streptomycin, and with a sensitivity value within the therapeutic concentration range usually attained. For these reasons we have adopted the disc of 10 μg. streptomycin for our standard test, and the graph is marked accordingly. Discs containing a greater amount of the antibiotic, e.g., 100 μg., may be used to cover a wider range of concentrations.

This standard test may be carried out in any routine laboratory without an appreciable increase in labour, and with either Lowenstein-Jensen, saponated blood agar, or similar medium. Only one bottle of the medium is required for the test, together with a bottle for control growth without a disc if desired. Both sensitive and resistant control tests are also set up with each series of tests. No special skill is necessary in setting up the test, and only a little extra time to that required for routine diagnostic culture. Moreover, the results are easy to read.

A most interesting observation is that the zones of inhibition of growth on saponated blood agar are sometimes smaller than with the same strain on Lowenstein-Jensen medium, and this may manifest itself only after the tenth day, when there is an apparent growing in of the organisms towards the disc on the saponated blood agar. This finding may be in agreement with those of Fisher (1952) and Mitchison (1953), who found that strains of *M. tuberculosis* resistant to isonicotinic acid hydrazide tend to lose the capacity for growing on simpler media, and require extra factors for growth. In such instances, resistance to a drug may only be shown on media that contain these factors; these may be present in saponated blood and not in Lowenstein-Jensen medium, and this may be correlated with our evidence showing saponated blood agar to be a much more conducive medium for the tubercle bacillus, lessening the generation time and so enabling the organism to adapt itself more rapidly to grow in the presence of streptomycin, for example, by the adoption of an alternative metabolism.

Alternatively, the difference in the zone of inhibition may illustrate streptomycin as a bacteriostatic, rather than a bactericidal, agent; this would occur more readily on saponated blood agar, where the growth of the organism is more favoured than on Lowenstein-Jensen medium.

Thus strains apparently resistant to streptomycin on saponated blood agar are probably resistant *in vivo* to the concentration covered by the test, though apparently fully sensitive on Lowenstein-Jensen or Dubos medium. It must be remembered that in such an *in vitro* test the results must be interpreted with caution, and are not necessarily a true reflexion of the interaction of the bacillus, host, and antibiotic. Up to the present, the correlation of *in vitro* sensitivity of the tubercle bacillus with the clinical result of therapy has not been of a very high standard (Gernez-Rieux, Sévin, and Chénet, 1949). It is possible that the results of tests on saponated blood agar may be more closely correlated with the clinical results of treatment, and this is under investigation.

Summary

A saponated blood agar medium is described for the rapid isolation of *M. tuberculosis*.

The tubercle bacillus was isolated from 115 consecutive specimens of sputa microscopically positive more rapidly on this medium than with Lowenstein-Jensen medium, the difference being more than 20 days in some cases. Fifty strains of *M. tuberculosis* subcultured on saponated blood agar grew more rapidly than when subcultured on Lowenstein-Jensen medium. In no instance did Lowenstein-Jensen medium give more rapid growth than saponated blood agar.

The streptomycin sensitivity of the tubercle bacillus was determined on saponated blood agar and on Lowenstein-Jensen media, the disc diffusion technique being used. This test may be carried out on cultures direct from the specimen, and simultaneously read along with the result of primary culture. Alternatively, the test can be carried out with pure cultures of tubercle bacilli.

The diameters of the zones of inhibition have been related to the inhibitory concentrations of streptomycin, and the sensitivity of each organism may be found by using the graph shown.

The significance of the results is discussed.

We express our thanks to Prof. T. J. Mackie for his continued interest and advice, and to Dr. A. T. Wallace for specimens of sputa.

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THE USE OF STREPTOKINASE BILE SALT BROTH FOR CLOT CULTURES IN THE DIAGNOSIS OF ENTERIC FEVER

BY

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In spite of the constant reiteration in almost every publication on typhoid fever that bacteriological examination of the blood is the diagnostic method of choice in the early stage of the disease, coupled with the steady stream of post-war publications emphasizing the uselessness of the Widal test at this time, it is remarkable how many requests for the latter are still made as the primary test and how few for the former. The determining factor is probably convenience, and although it has been possible to insist on blood cultures being done in hospital practice, it will be a long time before a similar routine can be effectively established in the mission outstations or in the field. A practical routine for the demonstration of the causative organism in the clotted blood specimen sent for the Widal test would turn a probably useless specimen into one of certain diagnostic value.

As early as 1906 Fornet demonstrated that clot culture was a practical procedure. We know that many tropical and sub-tropical laboratories use such a technique, as we have done in West Africa, India, and China, but the only references to it in the literature of the last 30 years appear to be those of Felix (1924) and Downie and Fairbrother (1934).

In the past it has been our practice to add the whole blood clot after separation of the serum (representing 4–5 ml. blood) to 10 ml. 0.5% sodium taurocholate broth in a screw-capped bottle, and, after vigorous shaking, to incubate and plate out on to Wilson and Blair's medium daily. A preliminary series of duplicate experiments showed that bile salt broth was preferable to "selenite" F broth, as the latter was inhibitory during the long incubation period required. At first the clots were incubated for 10 days before discarding, and the majority of those yielding a growth of *S. typhi* did so on the sixth or seventh day. Following the observations of Batty Shaw and Mackay (1951) that 10 days' incubation was insufficient for enteric blood cultures, we extended the period to 21 days and recovered positive clot cultures in the third week. This time lag, however, negatived the value of the examination from the clinical aspect, and often only served to determine the diagnosis in retrospect.

It was noted that even after 21 days' incubation more than half of the tubes still contained appreciable volumes of clot, and we considered it probable that bacteria were trapped in the clot and unable to grow, especially in those cases in which there was only a low degree of bacteraemia. Various methods of breaking down the clot were tried, such as trituration with sterile glass rods, mechanical shaking, and the addition of saponin from 0.1%–10% concentration, but none materially shortened the time of appearance of positive cultures. The increasing clinical use of the fibrinolytic streptococcal enzyme streptokinase suggested that it might prove of value here.

Effect of Streptokinase on Clot Cultures

Ten ml. volumes of bile salt broth were prepared with concentrations of streptokinase* varying from 25 to 10,000 units per ml. To each was added 3 ml. sterile blood clot, and these, together with control tubes without streptokinase, were incubated at 37° C. After 24 hours there was no apparent lysis in the control tubes or in those containing 25 and 10,000 units of streptokinase. The intermediate enzyme concentrations (50, 100, 250, 500, and 1,000 units per ml.) all showed partial lysis. Lysis was complete in these tubes at 36 hours and was partial in the 25- and 10,000-unit tubes, while the control tubes still showed no lysis. Five of the seven control tubes still showed appreciable clot after 14 days' incubation. We accepted 100 units per ml. as a suitable concentration for lysing the normal Widal clot in 36 hours, and used this concentration for all subsequent work. Streptokinase cannot be used in "selenite" F medium, as selenium inhibits enzyme action.

* The streptokinase used was kindly supplied by the Lederle Laboratories Division of the American Cyanamid Co. in the form of a white powder containing 100,000 units streptokinase and 25,000 units streptodornase per vial. A considerable amount of hyaluronidase is also present.

Effect of Streptokinase on S. typhi

Similar series of streptokinase–bile salt broth tubes were set up and inoculated with one loopful of a six-hour digest broth culture of *S. typhi*. Twenty-one recently isolated virulent strains together with the stock laboratory strains 901–0, Ty2, and Bhatnagar Vil were tested. After overnight incubation no inhibition of growth was found when compared with the control tubes with concentrations of streptokinase up to 250 units per ml. There was a slight inhibition at 500 units per ml. and complete inhibition at 1,000 units per ml.

Stability of Streptokinase in Bile Salt Broth

The inhibitory action of 1,000 units per ml. of streptokinase was used to estimate its stability in bile salt broth. Two series of bile salt broth tubes were prepared containing this concentration of streptokinase, one being left at room temperature (about 22° C.), and the other stored at 4° C. One bottle of each series was inoculated daily for 21 days, together with a control, with one drop of an overnight culture of *S. typhi*, and both were incubated overnight. In every case inhibition of growth in the streptokinase tubes was complete. The conclusion that there was no appreciable loss of enzyme activity over this period was confirmed by the more accurate assay technique described by Cathie (1949).

Recovery of S. typhi from Artificially Infected Clots

A number of artificially infected clots of 6 ml. volume were prepared, each containing approximately 100 organisms of a recently isolated strain of *S. typhi*. These were bisected under sterile conditions and the halves inoculated into 10 ml. bile salt broth and 10 ml. bile salt broth containing 100 units per ml. streptokinase respectively, each tube thus containing approximately 50 organisms embedded in 3 ml. blood clot. The tubes were incubated and plated out daily for 14 days on to Wilson and Blair's medium. Twenty-four such clots were tested with the following results:

Day	Streptokinase Bile Salt Broth		Bile Salt Broth	
	Positive	Negative	Positive	Negative
1	19	5	10	14
2	20	4	10	14
3	24	0	10	14
4	—	—	14	10
7	—	—	15	9
14	—	—	15	9

Thus 100% of the streptokinase broth cultures gave positive isolations within three days, the comparative figure for bile salt broth alone being 41.5%, while 37.5% of the latter were still negative after 14 days.

Viability of S. typhi in Blood Clot

Twenty-four artificially infected clots were prepared and bisected as above. One-half of each was inoculated into streptokinase broth, and *S. typhi* was recovered from these in every case. Of the remaining halves, 12 were stored at 37° C. and 12 at 4° C. for 14 days and then inoculated into streptokinase broth. *S. typhi* was recovered from all the refrigerated clots, but failed to grow from the 37° C. series within 14 days. It is probable, therefore, that part at least of the cause of our not being able to recover viable organisms from clots which have been sent by post or rail in the Natal climate lies in a direct action of the blood upon the bacteria. This point is being investigated further.

Application to Routine Diagnosis

To investigate the practicability of this method for routine diagnosis 350 clots from Widal specimens sent to this laboratory were cultured in parallel in plain bile salt broth and in bile salt broth containing 100 units per ml. streptokinase. The specimens are received in a sterile 75 × 12 mm. glass tube with a rubber bung. After separation of serum, the clots are decanted into a sterile petri dish and bisected with two sterile microscope slides, the halves being inoculated into 10-ml. volumes of the respective media. Daily subcultures for 14 days are made on to Wilson–Blair's bismuth-sulphite agar. By using a numbered template mounted on a sheet of glass illuminated from below and a 5-mm. loop, it is possible to spot 25 subcultures on to a standard 90-mm. plate. Positive cultures yielded a black ring of growth at the periphery of the spot with the typical metallic sheen after 24 to 36 hours' incubation (Fig. 1). All posi-

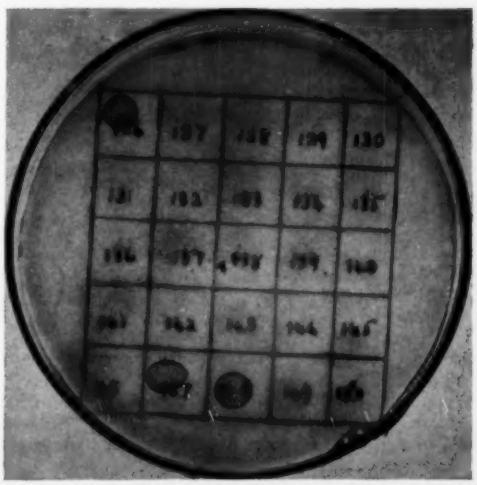

FIG. 1.—Three positive *S. typhi* spot cultures (126, 147, 148) on Wilson and Blair's medium spotted with 25 cultures.

tive or suspicious spots are subcultured, and the organism identified in the usual manner. It was found convenient to continue spotting positive cultures throughout the fortnight, as the position of the positive spot on the plate served as an additional check on the accuracy of plating. The whole investigation required nearly 10,000 spot cultures.

Typhoid is endemic in the native reserves of Natal, and normally some 300 isolations are made annually in this laboratory. Unfortunately, this investigation was made in the two winter months when the incidence is at its lowest, so that only 20 cases were proved bacteriologically during this period. The results, however, appear sufficiently striking to warrant reporting. Of the 20, one yielded a positive blood culture, showed an O titre of 640, but failed to give a positive clot culture in either of the two media. The blood culture and Widal specimens were taken at the same time. This is the only case in which a positive blood culture and negative clot culture has been observed. In one other case the clot was too small to split and was inoculated into streptokinase broth only, yielding a growth of *S. typhi* on the first day. Of the remaining 18, 16 yielded *S. typhi* and two *S. paratyphi A* (Table I).

TABLE I

COMPARISON OF POSITIVE ISOLATIONS IN BILE SALT BROTH WITH AND WITHOUT STREPTOKINASE

Case No.	Day of Positive Isolation		Organism	Widal Result	Blood Culture
	Streptokinase Broth	Bile Salt Broth			
19147	1	1	S. typhi	Neg.	Not done
23153	1	1	,, ,,	,,	,, ,,
22792	1	3	*S. paratyphi A.*	,,	,, ,,
21049	1	4	*S. typhi*	,,	,, ,,
21634	1	Neg. at 14	,, ,,	,,	,, ,,
20420	2	2	,, ,,	H+	+10th day
22122	2	2	,, ,,	Neg.	Not done
22155	2	2	,, ,,	,,	,, ,,
23448	2	2	,, ,,	,,	,, ,,
18754	2	Neg. at 14	,, ,,	H+	,, ,,
20810	2	,, ,, ,,	,, ,,	H+	Neg. 21 days
18505	3	3	,, ,,	H+	+11 days
19436	3	5	,, ,,	H+	+ 1st day
20419	3	5	*S. paratyphi A.*	AH+	+ 1st day
19661	3	Neg. at 14	*S. typhi*	Neg.	Neg. 21 days
19708	3	,, ,, ,,	,, ,,	,,	Not done
23154	3	,, ,, ,,	,, ,,	H+	,, ,,
21190	4	8	,, ,,	H+	,, ,,
19183	1	Not done	,, ,,	Neg.	,, ,,

Of the 18 clots, 17 were positive in the first three days in streptokinase broth, while only eight were positive in bile salt broth. The eighteenth was positive on the fourth day in the former; this clot was contaminated and a pure culture of *S. typhi* was only obtained on that day. In plain bile salt broth one became positive on the fourth day, two on the fifth and one on the eighth day, while the remaining six (33.3%) were still negative on the fourteenth day. Of the streptokinase isolations, the average time for a positive culture to appear was 2.1 days. Only two-thirds of the parallel bile salt cultures were positive, becoming thus at an average of 3.2 days.

The Widal tests were positive at a "diagnostic" titre in eight of the 19, the remaining 11 being negative. Parallel blood cultures in 35 ml. bile salt broth were sent in only six cases. Of these, two were positive on the first day, two on the tenth–eleventh day, and two negative on the twenty-first day. Visible clot was still present at this time. In both of the negative cases the bile salt clot culture was negative on the fourteenth day, while the streptokinase cultures were positive on the second and third day respectively.

Discussion

Although this series is small, we feel that the use of streptokinase bile salt broth for clot culture offers an additional practical technique for the positive bacteriological diagnosis of enteric fever. The causative organism was isolated within four days from the residual clot in 19 of 350 specimens sent for Widal examination, the latter being negative in eleven. In the two months before the present experiment, when clots were cultured in bile salt broth alone, 32 positive isolations were made from 346 specimens from clot cultures, 25 of these showing negative Widal reactions. The overall gain in diagnosis was thus 36 in the total of 50 isolations.

The action of streptokinase appears to be the purely enzymatic one of dissolving the clot rapidly and allowing the entrapped organisms free access to their nutrient medium. This is normally complete within 24 to 36 hours, so that positive cultures may be expected and occur within the first three days of incubation. This rapid result compares favourably with those of Batty Shaw and Mackay, who, in analysing 192 blood cultures in taurocholate broth, found the average time of incubation to yield a positive result was four to five days, and 17% did not become positive until nine to eleven days. The fact that blood clot itself exerts a bactericidal action on *S. typhi* at 37° C. is of importance, and emphasizes the need for rapid liberation of the organism from it. It is probable that this factor is responsible for the failures to isolate the organism in simple bile salt media from both clot cultures and blood cultures in which clotting has occurred, as the lytic action of sodium taurocholate is small. The addition of an anticoagulant such as sodium citrate or a lytic agent such as saponin to the commonly used bile salt blood culture media would appear to be mandatory.

The availability of chloramphenicol has emphasized the need for early diagnosis of enteric fever, and in hospital practice there can be no question

that early and, if necessary, repeated blood culture is the method of choice. Streptokinase broth offers a practical method of blood culture for domiciliary use and should materially assist in rapid diagnosis when only a clotted specimen of blood can be obtained.

Summary

Parallel cultures in streptokinase bile salt broth (100 units per ml.) and in plain bile salt broth have been made on the residual blood clot from 350 specimens submitted for a Widal test.

In 19 cases *S. typhi* or *S. paratyphi A* was isolated from streptokinase broth within four days, the average time to produce a positive result being 2.1 days.

Bile salt broth cultures yielded positive results in 12 cases after one to eight days' incubation (average 3.2 days), and remained negative after 14 days in six cases which gave positive streptokinase broth isolations. Similar results were obtained with artificially infected clots.

Streptokinase is stable in bile salt broth for at least 14 days.

The use of streptokinase bile salt broth offers a rapid means of diagnosing enteric fever in domiciliary practice when only a clotted blood specimen can be obtained.

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FIBRINOGEN AND THE SLIDE COAGULASE TEST

BY

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The advantages of using solutions of fibrinogen instead of plasma for carrying out the slide coagulase test were pointed out by Berger (1943) when he showed that their use avoided false positives due to naturally occurring staphylococcal agglutinins in rabbit and human plasma, in addition to the fact that these solutions retained their activity considerably longer. Using fibrinogen he demonstrated as complete a correlation between the slide and the tube method as had already been shown to exist between the two tests by Birch-Hirschfeld (1934) and Cadness-Graves, Williams, Harper, and Miles (1943) when fresh plasma is used for both tests. Berger prepared his solution from fresh citrate plasma by half saturation with sodium chloride for four hours in the ice chest, and dissolving the precipitate in sterile water to the volume of plasma originally taken.

While such preparations give good results and may retain their activity for several weeks if kept in the refrigerator, they tend to become infected with constant use, thereby losing their activity, and after being in use for a week or two they often become cloudy from precipitation of fibrinogen or its degradation products and the results of the test may be difficult to observe. The addition of a small crystal of thymol does not appear to interfere with the coagulase reaction and does seem to delay the onset of infection even if it does not stop the cloudiness and denaturation of the fibrinogen. Lastly, difficulty may be encountered at times with the precipitation of the fibrinogen, which may be delayed or incomplete.

Method

For over a year this laboratory has prepared and stored solutions of crude fibrinogen for the slide coagulase test by the following method:—

Plasma is one-fifth saturated with ammonium sulphate, and precipitation is usually complete at room temperature in from 10 to 15 minutes. The precipitate is then firmly thrown down by centrifuging at 2,500 r.p.m. for five minutes. The supernatant is poured off and the universal container allowed to drain for a few minutes, inverted on filter paper. The precipitate is then taken up in 10% aqueous urea to a volume roughly the same as the volume of plasma originally taken. The three stages of precipitation, centrifugation, and final solution and storage can be conveniently carried out in the same 20-ml. universal container. This gives a preparation faintly opalescent or as clear as water, and when it is brought into use the screw cap is replaced by a similar one penetrated by a teated glass pipette drawn out to a fine point for ease in delivering single drops on to the slide.

The source of plasma is usually the centrifuged, unused remains of blood from the bottom of transfusion bottles. Where this is not readily available, the method outlined here can be equally well applied to the unused remains of oxalated blood used for haematological or biochemical investigations, fluoride plasma for blood sugar estimation, or reconstituted plasma. Such specimens often require to be cleared of blood cells, debris, or fat, and the centrifuge may fail to do this, especially when dealing with samples of reconstituted plasma, but it may be satisfactorily carried out by Seitz filtration using a clearing pad. Duthie and Lorenz (1950) showed that this manoeuvre also removes most of the prothrombin but not fibrinogen or undue amounts of the accelerator factor, which was found by Smith and Hale (1944) to combine with staphylocoagulase to form a substance which will then convert fibrinogen to fibrin in the absence of calcium or thrombin. Such solutions will retain their activity for at least one month if kept on the bench away from direct sunlight, and for upwards of five months when stored in the refrigerator.

Storage

The use of aqueous urea as a solvent and for storage follows the observations of Meissner and Wöhlisch (1937), who refer to its effect on solutions of fibrinogen as a protective influence, and it is possible that the two substances form a loosely bound compound. In confirming this work, Diebold and Jühling (1938) found that although denaturation of fibrinogen does proceed in 10% urea, it is very slow, especially at 4° C., and the urea is considered by them as a preservative of the fibrinogen. Lastly, the denaturation products stay in solution so that the preparation retains its clarity while continuing to give a prompt clumping reaction with pathogenic staphylococci independently of the source of plasma used in the first instance. We have also found that 10% urea has a sufficiently strong

antibacterial action to suppress almost all accidental infection of the solution.

To demonstrate this, 2-ml. lots of fibrinogen in 10% urea were seeded separately with *Staphylococcus aureus* (Oxford) at 13 m. organisms per ml., *Bacterium coli* at 17 m. organisms per ml., and *Bacillus subtilis* at 6 m. organisms per ml. Tubes were left on the bench at room temperature, which varied between 24° C. and 27.5° C., and subcultures made at eight-hour intervals. Up to 24 hours subcultures showed heavy growth in all tubes, but after 72 hours all tubes seeded with *Staph. aureus* and with *B. subtilis* were sterile and those seeded with *Bact. coli* gave only isolated colonies after heavy inoculation of a blood agar plate. Finally it was found that all these tubes retained their coagulase activity apparently unimpaired at the end of this experiment.

Discussion

Attention has been drawn to these methods of preparing and storing fibrinogen because of the increasing use of the slide coagulase test that naturally follows the recent re-classification of the aerobic, catalase-positive, Gram-positive cocci by Shaw, Stitt, and Cowan (1951) at the National Collection of Type Cultures. These authors have not only confirmed the findings of previous writers from Cruickshank (1937) onwards that the demonstration of staphylocoagulase is the most reliable single criterion of pathogenicity in staphylococci, but they have made it a dominant feature of the classification, outmoding such criteria as pigment formation. Dealing with this feature alone, they find that 25% of pathogenic strains produce no pigment, while gold and yellow pigments are found in 38% and 7% of non-pathogenic strains. In this respect the now recognized disturbing effect of antibiotics on pigment production is particularly relevant, for organisms that have been subject to antibiotics are among the commoner strains of staphylococcus met with in hospital work to-day.

While the tube coagulase reaction will remain the more delicate test if done under standard conditions, the slide coagulase reaction is a valuable screening one and essential when as many as 20 strains of staphylococci may be encountered in the course of a morning's work. The use of crude fibrinogen solutions appears to be more than an interesting application of the original observations of Loeb (1903) and Much (1908), and has a special interest for those laboratories which have no ready access to an animal house, or where the amount of haematological work may not make available sufficient daily amounts of fresh plasma for a relatively large bacterial department.

Summary

A modification of Berger's method is described for the preparation of crude fibrinogen for the slide coagulase test, using ammonium sulphate for precipitation and aqueous urea as a reagent with better solvent and keeping qualities.

The literature is briefly reviewed.

I am indebted to Dr. W. A. Gillespie of the Bristol Royal Infirmary for helpful criticism and advice.

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AN ESTIMATION OF PLASMA VITAMIN A AND THE VITAMIN A ABSORPTION TEST

BY

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Following the adaptation of the Carr and Price (1926) colour reaction to the determination of the vitamin A content of the blood by McCoord and Luce-Clausen (1934), Chesney and McCoord (1934) reported impaired absorption of vitamin A from the intestine in coeliac disease, and devised the vitamin A absorption test as an index of the absorption of fat from the intestine. The chemical method was improved by Kimble (1939) and by May, Blackfan, McCreary, and Allen (1940), in each case a photoelectric colorimeter of the Evelyn (1936) type being employed in place of the earlier visual matching of the colours. The vitamin A absorption test has since been used by a number of workers in the United States, who confirmed the findings of low fasting plasma vitamin A levels and flat absorption curves in coeliac disease (Breese and McCoord, 1939; Clausen and McCoord, 1938; May *et al.*, 1940; May, McCreary and Blackfan, 1942; Adlersberg and Sobotka, 1943a), in cystic fibrosis of the pancreas (Blackfan and May, 1938), in sprue and idiopathic steatorrhoea (Adlersberg and Sobotka, 1943b; Ingelfinger, 1943; Cayer, Ruffin and Perlzweig, 1945; Darby, Kaser, and Jones, 1947), in congenital atresia of the bile duct (May *et al.*, 1940), and in colitis (Page and Bercovitz, 1943).

It was soon found, however, that low plasma vitamin A levels and flat absorption curves may occur in conditions other than those characterized by steatorrhoea, particularly in chronic infectious diseases, e.g., in tuberculosis (Breese, Watkins, and McCoord, 1942), in giardiasis (Katsampes, McCoord, and Phillips, 1944), in catarrhal jaundice (Breese and McCoord, 1940), in cirrhosis of the liver (Ralli, Bauman, and Roberts, 1941), in malnutrition, inflammatory lesions, and cretinism (May and McCreary, 1941), and in several diseases of the skin (Ruch, Brunsting, and Osterberg, 1946; Peck, Glick, Sobotka, and Chargin, 1943).

It is thus evident that the low plasma vitamin A level and flat absorption curve is not in itself diagnostic of steatorrhoea although both findings are invariably present when fat absorption is impaired. Ingelfinger (1943) considers that in assessing the low vitamin A level and flat absorption curve much is gained by a knowledge of the plasma carotene level, which is also low in steatorrhoea (Cayer *et al.*, 1945).

Our purpose in this communication is to present a method of plasma vitamin A estimation and to record data which we have found relevant to the performance and interpretation of the absorption test.

Method

Although the modifications of the Carr and Price blue reaction for the estimation of the vitamin A content of the plasma present little practical difficulty, these have in common several inherent drawbacks, viz., (1) the very rapid development and disappearance of the blue colour requires that the antimony trichloride reagent be added to the test solution in the photoelectric colorimeter, necessitating some technical dexterity; (2) the blue colour is not specific for vitamin A, being also given by the carotene compounds. Although this difficulty is to some extent overcome by the use of suitable filters or of a spectrophotometer, the methods in use generally require the application of a theoretical correction factor; (3) at low vitamin A levels the intensity of the blue colour is inadequate to permit sufficiently accurate determination; (4) a practical difficulty arises in the cleaning of cuvettes on which, if they are allowed to become wet, a white deposit forms. For these reasons we found the Carr and Price blue reaction to be inconvenient for routine clinical work and finally employed a modification of the spectrophotometric methods involving the destruction of vitamin A by ultra-violet irradiation (Bessey, Lowry, Brock, and Lopez, 1946; Karmarkar and Rajagopal, 1952).

Heparinized plasma (4 ml.), 8 ml. absolute alcohol, and 8 ml. *n*-heptane (Hopkin and Williams, " free from aromatic hydrocarbons "), are shaken in a mechanical shaker for 15 minutes. No troublesome emulsions are formed and the layers are allowed to separate. The heptane layer is pipetted off and divided into two approximately equal portions. One portion in a soda-glass

tube (12 mm. bore, 1 mm. wall and fitted with a glass stopper) is irradiated for three hours at a distance of 5 cm. from the Woods glass of a "hanovia" fluorescence type portable ultra-violet ray lamp. The optical density of the non-irradiated portion is determined at a wave-length of 327 mμ, using the irradiated portion for the blank setting, in a Beckman DU quartz spectrophotometer. The observed optical density is converted to international units vitamin A per 100 ml. plasma by reference to a standard curve constructed by plotting the difference between the optical densities of irradiated and non-irradiated dilutions of a sample of international standard vitamin A in heptane. The curve is a straight line and the following equation may be derived:

$E_{1cm.}$ for 1 i.u. vitamin A per ml. heptane = 0.048 at 327 mμ.

Thus optical density non-irradiated sample − optical density irradiated sample × 4,200 = i.u. vitamin A per 100 ml. plasma.

The length of time during which irradiation is carried out will vary with the type of lamp employed. In Fig. 1 it is shown that, with the ultra-violet lamp available to us, destruction of vitamin A is complete at three hours.

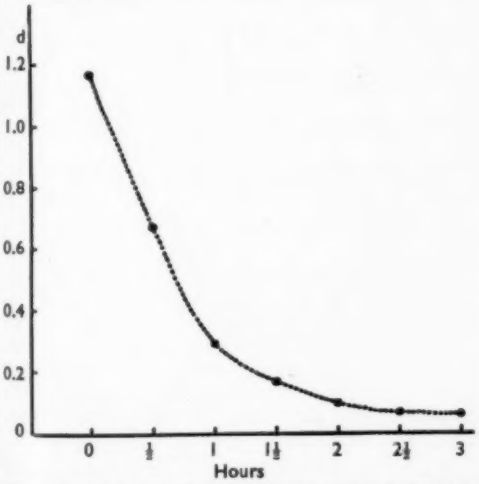

FIG. 1.—Destruction of a vitamin A solution in heptane by irradiation at a distance of 5 cm. from the Woods glass of a "hanovia" fluorescence type portable ultra-violet ray lamp. Ordinate: Optical density vitamin A solution at 327 mμ. Abscissa: Time in hours.

Vitamin A can be satisfactorily estimated by this method, which has also proved convenient for the following reasons: (1) Plasma and heptane extract (either before or after irradiation) may be kept at 4° C. for periods up to 48 hours without appreciable loss of vitamin A. (2) With experience, the amount of fluorescence of the extracts at the start of irradiation gives to the eye a rough indication of the vitamin A content of the samples, particularly when carrying out a vitamin A absorption test. (3) If the optical density of the heptane extract be determined using a heptane blank, and again after irradiation, 0.75 ml. plasma will suffice without resort to microestimation equipment.

The "Wetting" Factor in Vitamin A Absorption

One of the principal factors affecting the absorption of vitamin A from the intestine is the degree of "wetting" or dispersion of the vitamin A administered. In Fig. 2 are shown the variable

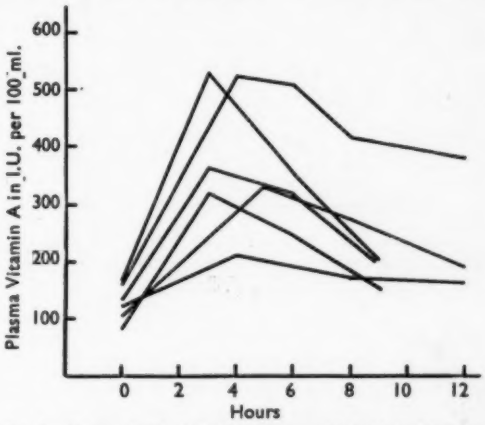

FIG. 2.—Plasma vitamin A levels in six normal individuals following 200,000 i.u. crystalline vitamin A orally, no attention being paid to whether the individual was fasting or not.

plasma vitamin A curves found in six normal individuals to each of whom a dose of 200,000 i.u. vitamin A ester in crystalline form was given in the morning without regard to whether breakfast was eaten, or, if eaten, the type of breakfast. In Figs. 3 and 4 the effect of prior solution of the vitamin in oil is evidenced by the much higher plasma levels reached in two normal individuals.

In recent years considerable experience has been gained both in experimental animals and in man (normal individuals and cases of steatorrhoea) of the comparative absorption of vitamin A administered in oily medium and in media in which the vitamin is in greater dispersion, viz. propylene glycol, dextri-maltose and the "tween" compounds (Lewis, Bodansky, Birmingham, and Cohlan, 1947; Kramer, Sobel, and Gottfried, 1947; May and Lowe, 1948, 1949; Jones, Culver, Drummey, and Ryan, 1948; Fox, 1949; Danielson, Binkley, and Palmer, 1949; Barnes, Wollaeger, and Mason, 1950). In general, absorption of vitamin A is greater and more rapid, faecal loss is diminished, and the plasma vitamin A levels are higher when vitamin A in these high-dispersion media is admin-

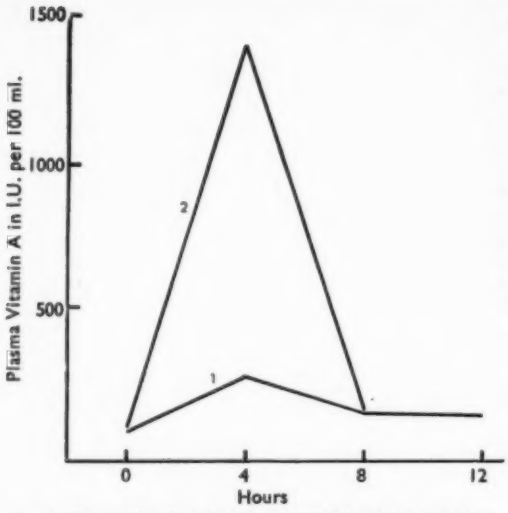

FIG. 3.—Plasma vitamin A levels in a normal individual (not fasting) (1) following 350,000 i.u. vitamin A in crystalline form orally, and (2) following 350,000 i.u. vitamin A in oil orally.

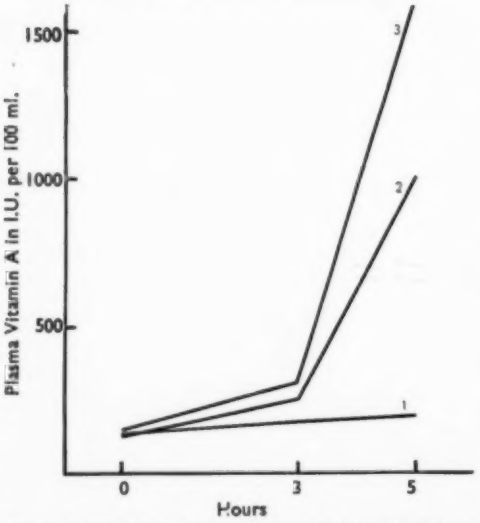

FIG. 4.—Plasma vitamin A levels in a normal individual (1) following 350,000 i.u. crystalline vitamin A orally fasting; (2) following 350,000 i.u. vitamin A in 1.5 ml. oil orally fasting; (3) following 350,000 i.u. vitamin A in 10 ml. oil orally not fasting.

istered to animals and to normal man. In pancreatic fibrosis there is an improvement in the absorption curve to near normal levels, but such improvement has been reported in only a few cases of coeliac disease, sprue, and idiopathic steatorrhoea (May and Lowe, 1949 ; Fox, 1949 ; Danielson *et al.*, 1949 ; Barnes *et al.*, 1950). In Table I are recorded the plasma levels of vitamin A in two cases of idiopathic steatorrhoea. In each case an absorption curve was determined after an oral dose of 350,000 i.u. vitamin A ester in oil. Polyoxethylene sorbitan mono-oleate (" tween 80 ") was given to the first patient for 10 days and to the second for four days in dosage of 6 g. per day. The absorption curves were then repeated, using the same dose of vitamin A in oil emulsified with sorbitan mono-oleate. It is seen that the effect of the emulsifying agent is to raise the fasting level and also that there is a slight but definite improvement in the absorption curve.

TABLE I

EFFECT OF POLYOXETHYLENE SORBITAN MONO-OLEATE ON THE VITAMIN A ABSORPTION CURVES IN TWO CASES OF IDIOPATHIC STEATORRHOEA

Patient	Method of Administration	Fasting	4 Hrs.	8 Hrs.	12 Hrs.
K. S.	Vitamin A in oil	43*	43	54	54
	Vitamin A in oil and polyoxethylene sorbitan mono-oleate	67	65	92	104
A. B.	Vitamin A in oil	46	102	74	45
	Vitamin A in oil and polyoxethylene sorbitan mono-oleate	69	125	95	86

* Figures are i.u. vitamin A per 100 ml. plasma.

Intermediate Absorption of Vitamin A

Emmet and Bird (1937) suggested that the naturally occurring esters of vitamin A were better absorbed than the free alcohol by the rat. Gray, Morgareidge, and Cawley (1940), however, demonstrated in the same species that hydrolysis of the esters occurred and that the alcohol form was present in the mucosal cells of the intestine. Drummond, Bell, and Palmer (1935) observed that after feeding the free alcohol to a patient with a fistula of the thoracic duct the vitamin was recovered from the chyle mainly as an ester.

In *in vitro* experiments with equal volumes of 90% methanol and heptane, vitamin A alcohol is equally distributed between the solvents, but vitamin A ester (palmitate) remains in the heptane layer. This method of separation (Popper, Steigmann, Dubin, Dyniewicz, and Hesser, 1948) was applied to the plasma obtained four hours after the oral administration of a dose of vitamin A alcohol. The heptane extract was shaken with 90% methanol but none of the vitamin A present in the heptane layer was found to go into the methanol layer. It seems probable, therefore, that vitamin A alcohol undergoes esterification during

the process of absorption. This may take place after the vitamin has been taken up by the mucosal cell.

In normal individuals and in patients with steatorrhoea neither Lewis *et al.* (1947) nor the present authors have found any significant difference between the absorption curves obtained after the administration of vitamin A alcohol or palmitate.

We have not, however, had the opportunity of making an adequate comparison in cases of pancreatic fibrosis.

Nature of the Vitamin A Absorption Curve

Barnes *et al.* (1950) have suggested that the height to which the plasma vitamin A level rises after oral dosage of vitamin A is dependent upon the degree of saturation of the vitamin A storage depots, or, in other words, that the test is a saturation or tolerance test. They therefore advocate the preliminary feeding of vitamin A for at least four days before carrying out the test in order to saturate the tissue stores. Popper, Steigmann, and Zevin (1943) have shown that massive doses of vitamin A orally did not alter the vitamin A absorption curve. This, too, has been our own experience both in normal individuals and in those with steatorrhoea. The absorption curve, however, must be the result of the rates of absorption from the intestine and of storage. It is thus possible that in vitamin A deficiency not due to an absorptive defect a very low vitamin A absorption curve would result.

Aron (1949) reviews suggestive evidence that there exists a mechanism which controls the level of vitamin A in the plasma, and shows that this mechanism may be upset in various disease processes. Thus it has been our experience that despite repeated experiments involving heavy dosage with vitamin A, our normal subjects maintained from day to day almost constant fasting plasma vitamin A levels. Aron considers that the liver plays an important part in the regulatory mechanism, and attributes the fall in the plasma vitamin A level seen in liver disease and in inflammatory processes to disturbance of the mechanism. It is not surprising, therefore, that it has been the experience of several investigators that the vitamin A absorption curve fails to reflect fat absorption in the presence of liver disease (Popper and Steigmann, 1943; Popper, Steigmann, and Zevin, 1943; Legerton, Texter, and Ruffin, 1953). As a rule the plasma vitamin A levels are low and the absorption curves flat. This is only in part due to the exclusion of bile from the intestine. Low levels and flat absorption curves are also seen in the presence of inflammatory disease.

In two cases of obstructive jaundice a yellow pigment has been extracted by the heptane from the plasma. This pigment has an absorption peak at 410 mμ and is broken down to colourless compounds by irradiation. These have a broad peak at about 300 mμ and interfere with the vitamin A extinction at 327 mμ. This is not carotene, which is stable in ultra-violet light, and we have been unable to find any reference to a heptane-soluble fraction of bile pigment. So far we have not been able to extract a similar pigment from bile obtained in the post-mortem room.

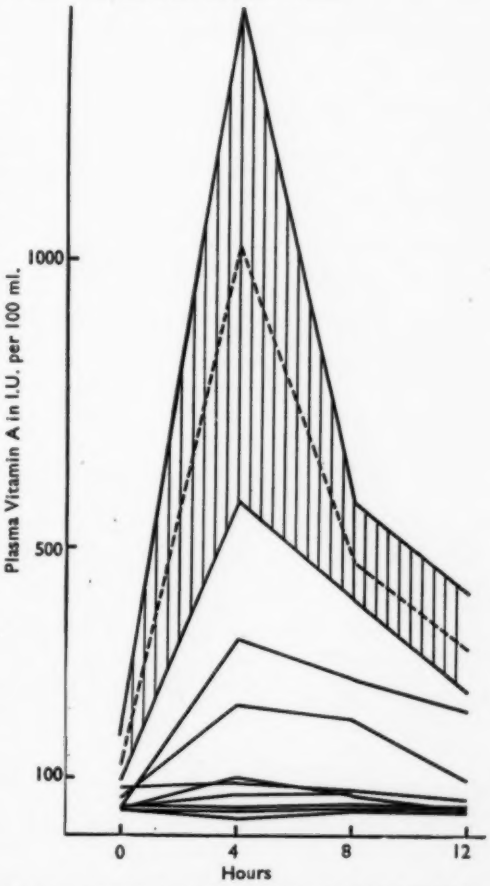

FIG. 5.—Plasma vitamin A levels in 11 normal individuals and eight patients with steatorrhoea following 350,000 i.u. vitamin A in oil (60,000 i.u. per g.) not fasting. Hatched area enclosed by the highest and lowest curves of 11 normal individuals, the dotted line being the arithmetic mean.

The Vitamin A Absorption Test in Steatorrhoea

The need for a standard procedure in carrying out the test is clear. Most investigators have administered the vitamin in the form of oleum percomorph (60,000 i.u. per g.) in dosage of 7,500 i.u. per kilogram body weight, with an upper limit of 350,000 i.u. We have done likewise, but have prepared solutions of crystalline vitamin A alcohol or palmitate in arachis oil in order to overcome the nausea which many individuals suffer after a dose of fish oil. To dissolve vitamin A in oil is difficult, and prior solution in a small quantity of alcohol is required. Since we felt that the object of the test should be restricted simply to determine whether vitamin A is or is not adequately absorbed from the intestine, we considered that no purpose is served in withholding meals. In Fig. 5 the highest and lowest plasma curves, together with the mean curve of 11 normal subjects, are drawn. Vitamin A absorption curves of eight cases of steatorrhoea, confirmed by fat balance studies, are also shown. These results are in close accord with those of Legerton *et al.* (1953) and earlier authors cited above, and permit the conclusion that although a flat vitamin A absorption curve is not necessarily diagnostic of steatorrhoea, the finding of a plasma vitamin A level of 500 i.u. or more between four and five hours following the oral dose of vitamin A in oil would almost certainly exclude a diagnosis of steatorrhoea. The vitamin A absorption test is thus a valuable screening procedure.

Summary

A spectrophotometric method utilizing the destruction of vitamin A by ultra-violet irradition has been employed for the determination of the plasma vitamin A level.

The vitamin A absorption test and factors which relate to its performance and interpretation are discussed.

A low vitamin A absorption curve is not diagnostic of steatorrhoea, but the test as described is of value in that adequate absorption of vitamin A excludes a diagnosis of steatorrhoea.

We wish to express our thanks to colleagues who acted as control subjects, and to Professors E. J. King and J. MacMichael in whose departments the work was carried out.

The samples of vitamin A were given to us by Roche Products Limited, and the sample of international standard vitamin A through the kindness of Dr. H. L. M. Perry, of the Department of Biological Standards, National Institute of Medical Research.

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A RAPID METHOD OF IDENTIFYING PARTICULAR BARBITURATE DERIVATIVES IN SMALL SAMPLES OF BLOOD BY PAPER CHROMATOGRAPHY

BY

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The quantitative estimation of barbiturates in blood by ultra-violet spectrophotometry (Goldbaum, 1948 ; Walker, Fisher, and McHugh, 1948 ; Born, 1949 ; Lous, 1950 ; Wright and Johns, 1953) is of limited value unless the particular barbiturate derivative is known or can be identified. Goldbaum (1952) described an ultra-violet spectrophotometric technique by which he claimed to make such identification, but many workers with experience in this field will prefer to rely on more direct methods of qualitative analysis. For general purposes the micro-crystalline method of Turfitt (1948) is suitable if urine or gastric contents are available, though even then it is always best to confirm the identification by extraction and mixed melting point determination. Cases of barbiturate poisoning frequently occur, however, where such material is not available for analysis, or if available, does not contain adequate amounts of barbiturate, as in poisoning by short-acting derivatives. It is in these cases that a qualitative method applicable to small blood samples is of particular value. Paper chromatography seems suitable for this purpose.

Wickström and Salvesen (1952) described three satisfactory solvent systems and were able to separate 10 barbiturate derivatives by paper chromatography. Algeri and Walker (1952) were apparently the first to apply paper chromatography to blood extracts, and they explored at least 27 solvents before settling on a butanol-ammonia system which enabled them to identify 10 barbiturate derivatives. These authors used chemical methods to reveal the spots, though Algeri and Walker employed ultra-violet light to show up thiopentone. Grieg (1952) sprayed the chromatogram with alkali and demonstrated the spots by holding the paper up to a fluorescent screen illuminated by ultra-violet light.

Paper chromatography is a relatively slow process if excellent results are to be obtained, and the present method sacrifices certain refinements in the interests of speed, since a delayed report is of little use to the clinician.

Principle

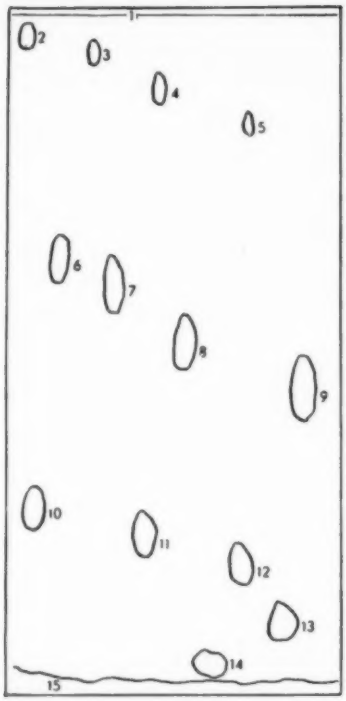
Barbituric acid derivatives are selectively distributed between suitably buffered filter paper and a chloroform moving phase.

Materials

Re-distilled chloroform, Whatman No. 1 filter paper strips, stock solutions of M-20 sodium carbonate and M-20 borax for making suitable buffers, and a "hanovia chromatolite" lamp are required.

Method

Descending chromatograms are run in a glass tank measuring $25 \times 25 \times 60$ cm. fitted with a ground-glass lid and kept in a dark cupboard. The atmosphere inside the tank is kept saturated with water and chloroform vapour, but owing to the liability of chloroform to decompose, the tank is washed out frequently. Stock mixtures of 0.05 M-borax and 0.05 M-sodium carbonate are used to make up buffers of *p*H 10 and *p*H 10.6 as required (Clark, 1928) and the *p*H of each buffer is checked on a lithium glass electrode before use. Rectangular strips of No. 1 Whatman filter paper 15×50 cm. are then sprayed with the appropriate buffer solution, usually *p*H 10.6, about 20 ml. of buffer being required to saturate this area of paper. The paper is left suspended in air by the edge which will ultimately hang lowest in the tank, and is used as soon as it feels reasonably dry. Paper which appears to have become thoroughly dry may be used provided that, after the application of the spots, it is allowed to hang in the moist atmosphere of the tank for at least an hour before the moving phase is added. Barbituric acid derivatives in 0.25% alcoholic solution are applied to the base line 2.5 cm. apart with a capillary pipette graduated to contain 0.01 ml. Small spots are obtained by intermittently applying the pipette to filter paper under an infra-red lamp. The paper is then suspended in the tank with its upper end immersed in re-distilled chloroform.



Key to Chromatogram	Official Name	Some Proprietary Names
1 Starting line		
2 Phenyl methyl barbituric acid		Rutonal (May & Baker)
3 Diethyl barbituric acid	Barbitonum (B.P.)	Veronal (Bayer) Medinal (Schering)*
4 Phenyl ethyl barbituric acid	Phenobarbitonum (B.P.)	Gardenal (May & Baker) Luminal (Bayer)
5 Diallyl barbituric acid	Allobarbitonum (B.P.C.)	Dial (Ciba)
6 Allyl isopropyl barbituric acid		Allonal (Roche)†
7 Ethyl-cyclo-hexenyl barbituric acid	Cyclobarbitonum (B.P.C.)	Phanodorm (Bayer)
8 Allyl isobutyl barbituric acid		Sandoptal (Sandoz)
9 Butyl ethyl barbituric acid	Butobarbitonum (B.P.C.)	Soneryl (May & Baker) Neonal (Abbott)
10 Isoamyl ethyl barbituric acid	Amylobarbitonum (B.P.C.)	Amytal (Lilly)
11 Ethyl (1-methylbutyl) barbituric acid	Pentobarbitonum (B.P.)	Nembutal (Abbott)*
12 Allyl (1-methylbutyl) barbituric acid	Quinalbarbitonum (B.P.)	Seconal (Lilly)
13 Ethyl (1-methylbutyl) thio barbituric acid	Thiopentonum (B.P.)	Pentothal (Abbott)
14 Methyl cyclohexenyl-N-methyl barbituric acid	Hexobarbitonum (B.P.)	Cyclonal (May & Baker)
15 Solvent front		Evipan (Bayer)

* These products are sodium salts.
† This product also contains amidopyrine.

FIG. 1.—Paper chromatogram showing resolution of 13 barbituric acid derivatives. The purity of these compounds was confirmed by melting point determination. (Paper buffered to *p*H 10.6, chloroform run for six hours at temperature 21° C. Total length of run 45 cm.)

Adequate development of the chromatogram takes between four and seven hours; owing to the relatively slow progress of the solvent front as it nears the lower end of the paper, development may usually be allowed safely to continue overnight if this is more convenient. At the end of this time the paper is removed from the tank, dried in air, and is ready within five minutes to be viewed under ultra-violet light. A "hanovia chromatolite" lamp emitting maximal radiation at 253.7 mμ is used, and the spots, which appear dark on a bright background, are for ordinary purposes ringed in pencil, though permanent records can be made by suitable exposure to ultra-violet light of photographic paper placed in direct contact with the chromatogram. The point of maximal density of the spot (usually its centre) is used for making measurements. Should there be any doubt regarding their identitiy as barbiturate the spots are eluted with 0.05% sodium hydroxide, buffered to *p*H 10, and the solution submitted to ultra-violet spectrophotometry. Experience showed that less material is required to give well-defined spots of slow running barbiturates such as barbital, phenobarbital, and methyl-phenyl-barbital. These are therefore applied in 0.083% solution. Well-defined spots are thus obtained with amounts of barbiturate varying from about 10 to 25 μg. For chromatography of blood extracts the final acid solution used for ultra-violet spectrophotometry is extracted with ether three times, the ether phase is evaporated to dryness and sufficient alcohol added to give a concentration of barbiturate estimated at about 0.25%. Crude extracts of blood made by direct shaking with ether have also been used with success, though it is difficult to obtain spots of suitable size unless the approximate quantity of barbiturate present is known.

Results

Fig. 1 shows the distribution of 13 barbiturate derivatives run simultaneously on the same piece of paper. It will be seen that in general there is a relationship between the distribution of the various derivatives on the chromatogram and their relative speeds of clinical action; the slow acting barbiturates travel slowly, medium acting barbiturates more rapidly, and fast acting barbiturates most rapidly. The chromatogram thus parallels Tatum's (1939) classification of barbiturates, the only notable exception being ethyl cyclohexenyl barbiturate acid (cyclobarbitone "phanodorm") which this author classifies as short acting and yet runs relatively slowly on the chromatogram.

TABLE I

R VALUES RELATIVE TO PENTOBARBITAL (*p*H 10.6 CHLOROFORM)

	Mean	S.D.	Range	No. of Observations
Barbital ..	0·12±	0·06	0·06–0·21	10
Phenobarb ..	0·13±	0·01	0·10–0·17	11
Allyl isopropyl	0·61±	0·05	0·53–0·70	10
Butobarbital ..	0·69±	0·04	0·63–0·75	13
Amylobarbital	0·93±	0·03	0·88–0·98	15
Quinalbarbital	1·06±	0·02	1·01–1·08	9

This parallelism between the chromatographic distribution and clinical action is most useful from the diagnostic standpoint, since even if a given barbiturate derivative cannot immediately be identified, its position on the paper roughly indicates its speed of action so that an estimate can be made of the significance of blood levels already obtained by spectrophotometry.

As might be expected in a chromotographic system involving the use of buffered paper with chloroform as the moving phase, in which no great pains are taken to ensure uniform temperature or humidity, absolute Rf values are too variable to be useful in identifying particular derivatives. R values relative to marker barbiturates are of more use, particularly in differentiating derivatives which lie close to the marker. Table I shows some R values relative to pentobarbital. It is clear that amylobarbital and quinal barbital may readily be differentiated whereas the ranges of allyl-isobutyl barbital and butobarbital overlap as also do those of barbital and phenobarbital.

In practice the identification of an unknown barbiturate is best done by running it on the same sheet of paper as known representatives of slow, medium, and quick acting derivatives. Two alcoholic solutions of markers are used here, one containing phenobarbital, butobarbital, and pentobarbital and the other barbital, allyl-isobutyl barbital, and amylobarbital.

The slower running compounds can be more widely separated by running them on paper buffered to *p*H 10.0 or alternatively, using N butanol as solvent and paper buffered to *p*H 10.6.

Figs. 2, 3, and 4 are examples of unknown drugs identified by the above technique. Fig. 4 is of particular interest in that it shows how amylobarbital and quinal-barbital may be separated in the blood of a patient who had taken a mixture of

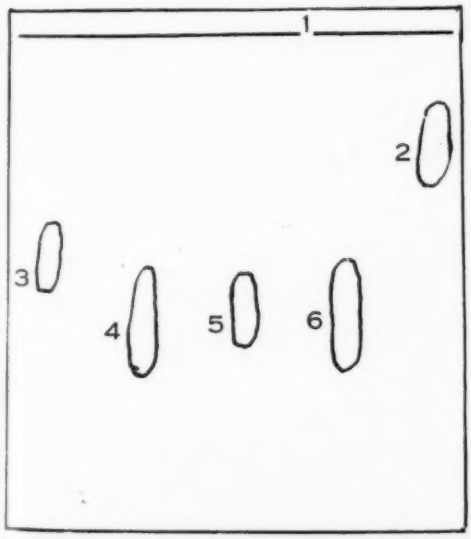


FIG. 2.—(1) Base line, (2) phenyl methyl barbituric acid, (3) diethyl barbituric acid, (4) unknown blood extract, (5) phenyl ethyl barbituric acid, (6) unknown blood extract. Conclusion.—4 and 6=phenyl ethyl barbituric acid.

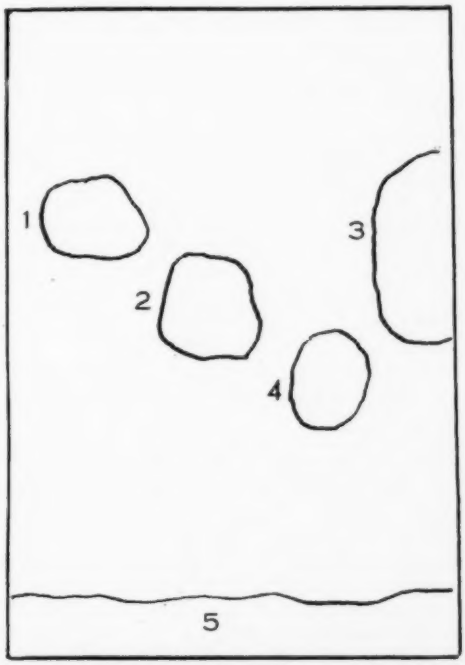


FIG. 3.—(1) Ethyl isoamyl barbituric acid, (2) ethyl (1-methylbutyl) barbituric acid, (3) unknown blood extract, (4) allyl (1-methylbutyl) barbituric acid, (5) solvent front. Conclusion.—Either the unknown blood contained both ethyl isoamyl and ethyl (1-methylbutyl) derivatives, or only one of these derivatives, the spot being too big. (Ethyl isoamyl barbituric acid was isolated from stomach contents.)

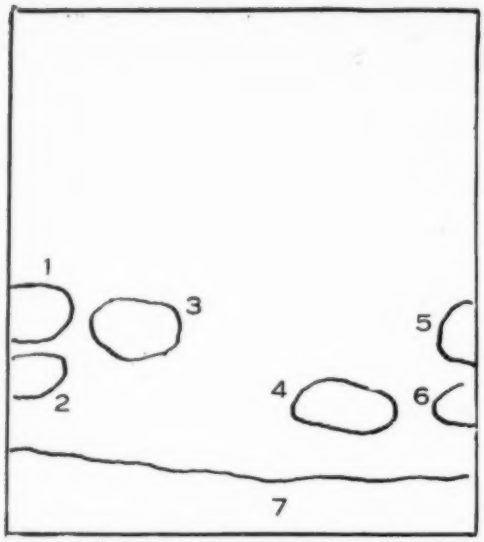


FIG. 4.—(1) and (2) Spots from extract of unknown blood, (3) ethyl isoamyl barbituric acid, (4) allyl (1-methylbutyl) barbituric acid, (5) and (6) spots from extract of stomach contents of same case as 1 and 2, (7) solvent front. Conclusion.—1 and 5=ethyl isoamyl barbituric acid; 2 and 6=allyl (1-methylbutyl) barbituric acid.

the two ("tuinal"). Altogether the barbiturates concerned in 20 cases of poisoning have been identified by this technique and in some cases successful identification was possible on blood specimens which had been stored in a refrigerator for periods as long as 18 months. Chromatographic identification of barbiturates present in urine, gastric contents, and tissues has also been made in a few cases.

Discussion

The distribution of barbituric acid derivatives in this system presumably depends on their individual pKs and also on their solubility in chloroform. Bush (1937) gave pK values for five barbiturate derivatives as follows: phenobarbital 7.34, barbital 7.89, amylobarbital 7.89, pentobarbital 8.04 and hexobarbital 8.23. These figures indicate a trend towards higher pK values in the more rapidly running compounds, as would be expected on theoretical grounds, though the fact that amylobarbital and barbital have the same pK value clearly indicates that their wide separation on the chromatogram is due to another factor. Tabern and Shelberg (1933) found that the barbiturate derivatives decreased in water solubility and increased in oil/water distribution coefficient with increase in length of the 5.5 substituted alkyl side-chains, and their data were used by Fuhrman and Field (1943) in reviewing the relationships between the physical properties and physiological actions of a number of barbiturate derivatives. Butler (1950), using mice, showed that the difference in the speed of action of a slowly acting derivative (barbital) and a rapidly acting one (ortal) could be accounted for by the relatively slow appearance of the former substance in brain tissue. He postulated that the essential difference in physiological action between barbiturate derivatives lies in their relative ability to transgress the brain cell membrane. Brodie (1952) has shown that the extreme rapidity of action of thiopental can be attributed to its high lipoid solubility rather than to chemical instability.

These researches indicate that barbiturates obey the Meyer-Overton hypothesis, so long known to hold for volatile anaesthetics. The present observations on the chromatographic distribution of 13 barbituric acid derivatives suggests that their relative solubilities in chloroform parallel their lipoid solubilities. The position of ethyl-cyclohexenyl barbital (cyclobarbital) is interesting, for this substance was classified by Tatum as rapidly active. According to the chromatogram it should have a slow medium action akin to butobarbital, and this is supported by clinical experience of this drug. In contrast, however, the analogue of this substance N methyl cyclo-hexenyl-methyl barbituric acid ("evipal") is one of the quickest acting barbiturates and indeed it runs almost with the solvent front with the system described here.

Algeri and Walker (1952) mentioned that with their system the spots obtained in some cases from blood samples of patients who have taken known barbiturates did not correspond with the position of the pure substance run on the same piece of paper. They interpret such differences as possibly due to metabolic products of barbiturate in the blood samples. With the present method no convincing differences of this nature have been obtained, the slight divergences which are sometimes observed being readily attributable to the size of the spot, irregularities in the texture or impregnation of the paper with buffer, or to slight asymmetry in suspension of the paper.

Summary

A simple, relatively rapid, paper chromatographic system is described for the separation and identification of barbiturates in small blood samples.

At least 13 of the common barbiturate derivatives can be separated by this means and their relative distribution correlated with their speed of clinical action.

I am very grateful to Dr. and Mrs. C. J. O. R. Morris for their valuable advice and to Professor Clifford Wilson for his encouragement and constructive criticism. Messrs. May & Baker kindly supplied the 13 barbituric acid derivatives used in this work.

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THE SERUM VISCOSITY AND THE SERUM PROTEINS

BY

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Equal weights of the various protein fractions in plasma affect the plasma specific gravity equally, whereas weight for weight the fractions exert differing influences on the plasma viscosity. Thus fibrinogen has a greater effect on the plasma viscosity than does globulin, which, in turn, has a greater effect than albumin (Bircher, 1921 ; Naegeli, 1931 ; Oberst, 1939 ; Cowan and Harkness, 1947). Working with mixtures of albumin and globulin, Naegeli (1931) and Nugent and Towle (1935) have shown that at a constant total protein content the viscosity of the mixture falls as the ratio of albumin to globulin rises, and vice versa.

It was considered that perhaps, given the total protein content and the viscosity of a serum, it might be possible to calculate the albumin : globulin ratio. Thus, using the copper sulphate specific gravity method for estimation of the total serum protein level (Harrison, 1947) and a modified Ostwald viscometer for the estimation of the serum viscosity (Lawrence, 1950), the estimation might be completed in under 15 minutes per serum, a much more rapid test than either the Kjeldahl or the Biuret methods. The specific gravity of a serum can be estimated to ±0.0005 (±0.18 g.%), and the serum viscosity ratio is accurate to ±0.2% (Lawrence, 1950). In fact, the estimation of the albumin : globulin ratio by this proposed method was not achieved.

Method

Blood, 7–10 ml., was taken by venepuncture from various patients and normal controls, allowed to clot, and the serum then removed. The total serum protein level and the albumin : globulin level were estimated by the micro-Kjeldahl method described by Harrison (1947). The serum viscosity was estimated by the method described by Lawrence (1950). Each serum was progressively diluted with physiological saline, and the viscosity of each dilution was estimated. From the curve obtained by plotting the total serum protein levels against their respective viscosities it was possible to calculate the viscosity of each serum at a constant total protein level. For convenience, this arbitrary level was taken as 6.5 g.%. Saline was used as diluent so that the various protein fractions should remain in solution (Bingham, 1945).

Also, a normal serum and serum from a case of advanced cirrhosis of the liver were mixed in varying proportions, and the viscosities of the various dilutions of the sera and the mixtures were estimated. Thus both the albumin : globulin ratios and the proportions of the various globulin fractions were varied in the mixtures.

Results

In Fig. 1 the viscosities of the two sera and their mixtures are shown at various dilutions. The

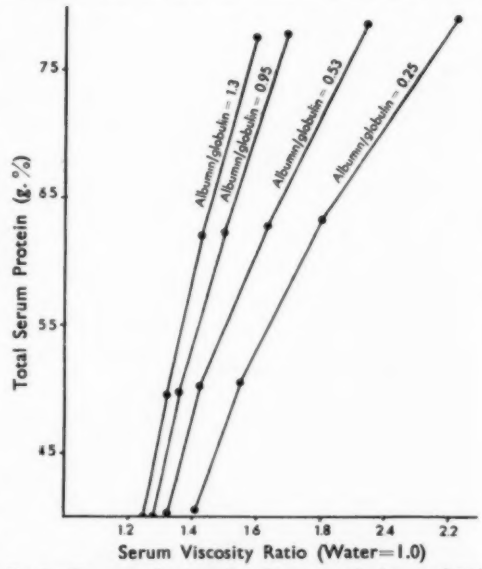

FIG. 1.—The serum viscosity ratios of two sera and mixtures, plotted against the serum protein concentration.

results resemble the graphs of Naegeli (1931) and Nugent and Towle (1935), in that as the albumin : globulin ratio rises so the serum viscosity falls.

Thirty-five results are shown in Fig. 2, in which the serum viscosity of each serum at a total pro-

tein content of 6.5 g.% is plotted against its corresponding albumin : globulin ratio. The sera were taken from two normals and from a number of patients with rheumatoid arthritis (nine), congestive cardiac failure (seven), multiple myelomatosis (three), carcinomatosis (two), unexplained anaemia in old age (two), advanced hepatic cirrhosis (one), lymphoma (one), diabetes with nephrosclerosis (one), acute rheumatic fever (one), dermatomyositis

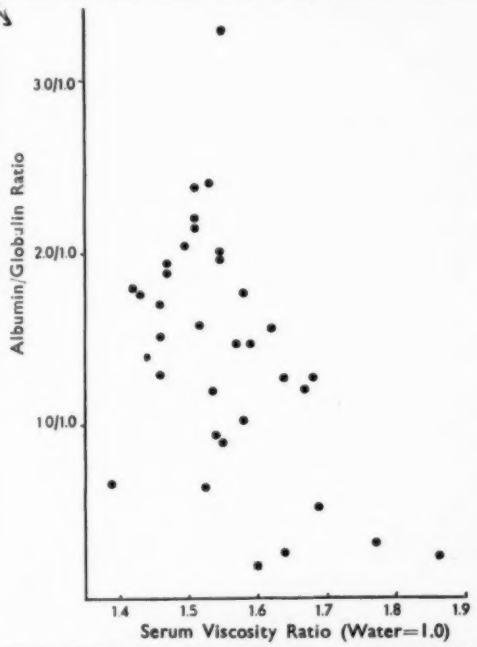

FIG. 2.—The albumin : globulin ratios of sera plotted against their serum viscosity ratios at a total protein concentration of 6.5 g.%.

(one) and pooled sera (five). Although, in general, the serum viscosity increases as the albumin : globulin ratio falls, there is a wide scatter in the readings of the ratio at any given serum viscosity value. Thus the estimation of this ratio from the serum viscosity and the total serum protein concentration is not feasible.

Discussion

Although the molecular weight of globulin is given as 155,250 by Harkness and Whittington (1947), they state that this is an average estimation. Globulin consists of many fractions, and Wuhrmann and Wunderly (1947) give the molecular weights of alpha-globulin as 500,000, beta-globulin as 2,000,000, and gamma-globulin as 150,000. It is reasonable to suppose that these fractions will have different effects, weight for weight, on the serum viscosity. Harkness and Whittington (1947) showed that a mixture of albumin and globulin also contained a combined (albumin-globulin) complex with a molecular weight somewhere between those of its components, and hence with an intermediate effect on the viscosity of the mixture. This being so, it is obvious that variations in the proportions of the globulin fractions, quite apart from any variations in the albumin : globulin ratio, will affect the serum viscosity. This would account for the wide scatter apparent in Fig. 2, and the resulting gross inaccuracy were the serum viscosity taken as a measure of the albumin : globulin ratio : it has a direct bearing on the erythrocyte sedimentation rate. It is known that the erythrocyte sedimentation rate depends on the aggregating action of the plasma, which is a complex function of the plasma viscosity (Whittington, 1942). Also globulin is known to have a direct effect on the sedimentation rate, although not as great as the effect of fibrinogen (Ley, 1922 ; Linzenmeier, 1923 ; Naegeli, 1931 ; Bendien and Snapper, 1931 ; Vogt, 1941). Aldred-Brown and Munro (1934) could find no close correlation between the sedimentation rate and the plasma globulin content or the albumin : globulin ratio, and from Fig. 2 it may be concluded that sera with the same viscosity will probably not influence the erythrocyte sedimentation rate to the same degree, since the viscosity may be the result of varying proportions of protein molecules of various sizes.

Ehrström (1939) suggests that euglobulin accelerates the sedimentation rate ; also it has been shown that euglobulin has a greater effect on the erythrocyte sedimentation rate than has pseudoglobulin, and that albumin positively depresses the sedimentation rate (Gray and Mitchell, 1942 ; Gordon and Wardley, 1943). Shedlovsky and Scudder (1942) found a direct qualitative relation to exist between the plasma alpha-globulin and the sedimentation rate, but not between either the beta-globulin or gamma-globulin fractions and the sedimentation rate, while Hardwicke and Squire (1952) found that alpha-globulin and gamma-globulin fractions were the most active of the globulin fractions in increasing both the sedimentation rate and the plasma viscosity. A further complication is suggested by the work of Kunkel and Tiselius (1951) ; using a two-way electrophoresis technique, they found that some plasma protein fractions, in particular the gamma-globulins, may often be heterogeneous. Thus, while the serum viscosity in conjunction with the total protein content will indi-

cate a qualitative change in the serum proteins, an accurate assessment of the nature of the globulins present or the albumin:globulin ratio is not possible.

Summary

Although sera with a high albumin:globulin ratio are less viscous than those sera in which the ratio is low, no simple relation has been found to exist between these two variables. Thus it is not possible to calculate the albumin:globulin ratio accurately from the total serum concentration and the serum viscosity.

This lack of correlation is probably explained by the different effects of the various globulin fractions on the serum viscosity, and by the possibility that some of these fractions may be heterogeneous.

The significance of these findings is discussed with relation to the effect of the plasma globulin on the sedimentation rate of erythrocytes.

I am indebted to Professor M. Maizels for encouragement and helpful criticism, and to Dr. J. S. Lawrence for the loan of one of his viscometers. The substance of this paper formed part of a thesis submitted for the degree of M.D. (Cambridge).

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TECHNICAL METHOD

A DIRECT DISC TECHNIQUE FOR DETERMINING THE SENSITIVITY OF ORGANISMS TO THE SULPHONAMIDES

BY

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The laboratory control of chemotherapy is becoming increasingly important for two reasons: (1) the majority of common infections may be caused by a wide variety of organisms, the nature of which cannot be determined by clinical methods; and (2) members of individual bacterial species or groups are tending to vary considerably in their reactions to the different agents. It is, however, necessary that laboratory tests should be relatively simple and give rapid and reliable information if they are to provide a useful control for routine purposes.

In the case of the antibiotics, these criteria have been largely fulfilled and laboratory control of therapy is widely used. With the sulphonamides, however, the practice is still restricted, due mainly to technical difficulties.

Laboratory tests with sulphonamides are greatly influenced by many factors, in particular the composition of the medium, the concentration of the organisms, and the period of incubation. The significance of the tests may also be uncertain, as marked discrepancies between *in vitro* and *in vivo* results do occur. Nevertheless sensitivity tests can offer useful information provided the conditions of the test are carefully standardized.

Several methods are available, e.g., the serial dilution technique and various forms of agar-diffusion, such as the ditch-plate, streak-plate, whole-plate, and the use of discs. The disc technique, at present used for the sulphonamides in some laboratories, is different from that used for the antibiotics; it is a two-stage method in which the discs are placed on the medium for a fixed period, after which they are removed and the plates seeded with the organism to be tested (Evans, 1948; MacFarlane, 1951).

The antibiotic disc test is a simple one-stage technique and, as it has given excellent results in routine practice, it was decided to investigate the possibility of adapting this to the testing of the sulphonamides.

Dried discs and tablets were tried and the following sulphonamides examined:—Sulphathiazole, sulphadimidine ("sulphamezathine" by Imperial Chemical (Pharmaceuticals), sulphafurazole ("gantrisin" by Roche) and a sulphasomidine ("elkosin" by Ciba).

Preparation of Materials

Medium.—It is essential in comparative work with the sulphonamides that all media should be standardized and that any sulphonamide-antagonizers should be neutralized. In consequence, lysed horse-blood agar was used throughout the investigation (Harper and Cawston, 1945; Walker, Philip, Smyth, and McLeod, 1947). The supernatant plasma of oxalated horse-blood was replaced by sterile distilled water and the reconstituted cell suspension well shaken; lysis was completed by alternate freezing and thawing, and the solution was stored in the refrigerator. The final medium was prepared by the addition of the solution to melted nutrient agar to give a final concentration of 5%.

Sulphonamide Solutions.—The sulphonamides are relatively insoluble compounds but, in the case of sulphathiazole and sulphadimidine, suitable dilutions were readily prepared from the soluble intravenous products. Sulphafurazole and "elkosin" were only available in the crystalline or powder form and the required solutions in distilled water were effected by boiling. In all cases a stock solution (1 g. in 10 ml.) was prepared and stored at room temperature; some recrystallization occurred on cooling with sulphafurazole and "elkosin" but this deposit re-dissolved on boiling, and further dilutions were then made as quickly as possible.

Discs.—Discs of 8 mm. diameter were punched from blotting-paper (Ford Mill 428); a different colour was used for each sulphonamide. After sterilization in the hot-air oven, a standard drop (50-dropper) of the four different sulphonamide solutions (1 in 80 final dilution) was added to each disc, giving a total amount of 0.25 mg. per disc.

The discs were stored in sterile universal containers or Petri dishes. They maintained their potency for a considerable time; chemical assays were made at

intervals up to five months and the discs proved very stable. There was no deterioration.

Tablets.—The tablets were prepared by Messrs. Evans Medical Supplies Ltd. Original supplies proved unsatisfactory as they gave irregular results and were unsuitable for use in sensitivity tests. The difficulties of preparation were apparently overcome, as later preparations were subjected to rigorous trial and proved quite consistent, giving uniform results by biological and chemical tests.

Tablets containing 1, 2, and 10 mg. sulphonamide were tried, but it was found that the zones of inhibition given by the 10-mg. tablet were only slightly larger than those produced by the 1-mg. tablets. Chemical assays of the tablets before and after incubation indicated that while the 1-mg. tablets lost approximately 60% of the sulphonamide, only about 20% diffused from the 10-mg. tablets. It was decided to use the 1-mg. tablets for the tests.

The Inoculum (Bacterial Suspensions).—All organisms were recently isolated from infective processes. It is generally accepted that the concentration of the inoculum exercises a great influence on the final result and that the degree of dilution varies with different organisms. As a rule the organisms were seeded into broth and, after six hours' incubation at 37° C., tenfold dilutions (up to 1 in 10^8) were made in sterile distilled water. The more fastidious organisms, such as pneumococcus, *Strept. haemolyticus*, and meningococcus, were grown in special media, e.g., O'Meara and Brown (1936), and serum broth, for 24 hours before dilution.

Sensitivity Tests

Ditch-plate Method.—Ditches were cut in the lysed blood agar and filled with 5 ml. of similar media containing the various concentrations of sulphathiazole and sulphadimidine. In preliminary trials a wide range (0.6–10 mg.) was used, but eventually a sulphonamide strength of 1.25 mg. per 5-ml. ditch was adopted for the investigation.

The plates were left for six hours at 37° C. or overnight in the refrigerator before use in order to allow adequate diffusion of the sulphonamide from the ditch. They were then seeded by streaking across the ditch with a standard loop (2.5 mm. diameter). Ten or twelve streaks were made on each plate, and at least four dilutions of each organism were tested. A known sensitive organism was invariably used as a control. Readings were made after incubation at 37° C. for 18 hours; the dilution producing discrete colonies was that used for making the final assessment of sensitivity to the drug.

The most suitable dilutions for this technique varied with the different organisms; those mainly used in this investigation were 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}. High dilution was essential for most organisms, but it is interesting to note that this had little effect on the results given by *Staph. aureus*.

The "Whole-plate" Method.—Plates containing 15 ml. of the lysed blood-agar plus the sulphonamides were prepared. The sulphonamides used were sulphathiazole and sulphadimidine in concentrations of 25 and 12.5 mg. per 100 ml. The plates were stored in the refrigerator, usually overnight, and after thorough drying were seeded in prescribed areas with a standard drop of the tenfold dilutions of the bacterial suspension; a control plate, free from sulphonamide, was always prepared for each test, and a known sensitive strain was used as a control organism. After incubation at 37° C. for 18 hours readings were made. Important factors in the final assessment of sensitivity were the differences in growth on the control and test plates, particularly with the dilutions producing discrete colonies on the control plate.

The Dried Disc Method.—The technique was similar to that used for the examination of the antibiotics (Fairbrother and Martyn, 1951). Pour-plates with appropriate dilutions of the bacterial suspensions were prepared on the lysed blood medium; after the excess of culture had been pipetted off the discs were placed on the surface of the medium at convenient intervals and the plates incubated at 37° C. for 14 to 18 hours. Readings were then made; the degree of sensitivity was assessed by the zone of inhibition around the discs.

Chemical analysis of the discs, before and after incubation, showed that 90% of the sulphonamides had been lost, probably by diffusion into the medium during this period.

Many dilutions of the organisms were investigated during the preliminary trials, and it was found that, while high dilution was usually necessary, the optimal strength varied with the different organisms. The following dilutions tended to give satisfactory results and were adopted for the trial: *Staph. aureus*, coliforms, and Friedlander's bacillus = 1/2,000 (six-hour culture); *Proteus* = 1/20,000 (six-hour culture); *Ps. pyocyanea* = 1/100,000 (six-hour culture); *Pneumococcus* and *Strept. haemolyticus* = 1/500 (24-hour culture).

Tablet Method.—The technique was similar to that of the disc method, with tablets of sulphathiazole and sulphadimidine substituted for the discs.

Results

The investigation was divided into two parts: (1) A comparison of the four different agar diffusion techniques using sulphathiazole and sulphadimidine; (2) a trial by the dried disc technique of four potent sulphonamides: sulphathiazole, sulphadimidine, sulphafurazole, and "elkosin."

The first part of the investigation was made with 190 organisms freshly isolated from routine material of diverse origin. Each organism was tested simultaneously by the four methods under comparable conditions; the tablets and discs were added to the same pour-plate (Fig. 1). The results are given in Table I.

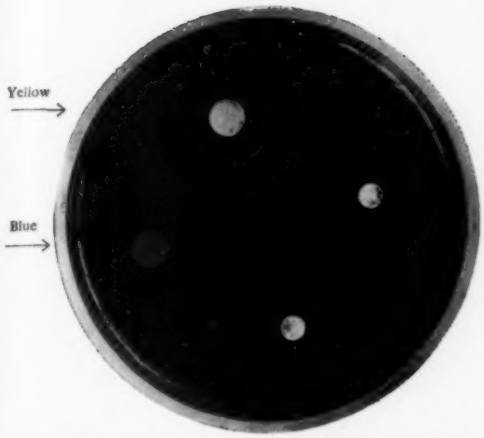

FIG. 1.—*Staph. aureus* tested against sulphathiazole (a yellow disc) and sulphadimidine (a blue disc) by the disc and tablet techniques. (Disc and tablet of the same drug are adjacent.)

TABLE I

SENSITIVITY TESTS ON 190 ORGANISMS BY FOUR DIFFERENT METHODS

Organism	Number Tested	Number Sensitive to Sulphathiazole				Number Sensitive to Sulphadimidine			
		Ditch Plate	Whole Plate	Disc*	Tablet*	Ditch Plate	Whole Plate	Disc	Tablet
Staph. aureus ..	75	52	52	52	52	48	48	48	48
Strept. haemolyticus ..	24	18	18	18	18	15	15	15	15
Strept. faecalis ..	11	0	0	0	0	0	0	0	0
Pneumococcus ..	10	10	10	10	10	10	10	10	10
N. meningitidis ..	4	4	4	4	4	4	4	4	4
Bact. coli	50	37	37	37	37	8	9	10	9
Ps. pyocyanea ..	16	9	9	9	9	0	0	0	0
Total ..	190	130	130	130	130	85	86	87	86

* In the disc and tablet techniques sensitivity is equivalent to a zone of inhibition at least 15 mm. in diameter.

The results of the four different methods were remarkably consistent. With sulphathiazole there was complete agreement, while in the case of sulphadimidine a very slight difference was observed only with the coliform group. Sulphathiazole was more active than sulphadimidine, particularly against the Gram-negative bacilli. It is interesting to note the irregular behaviour of individual members of some species or groups to both sulphonamides; only strains of the pneumococcus, meningococcus and *Strept. faecalis* gave completely consistent results. It follows that even though the identity of an organism has been established, this may not indicate the reaction to the sulphonamides of the particular strain under investigation.

A further series of 120 organisms was tested against four active sulphonamides by the dried disc technique (Table II). The discs were placed on the same pour-plate of the organism and thus the conditions of the test were strictly comparable (Fig. 2).

Readings were obtained without difficulty and, except in the case of *Ps. pyocyanea*, the

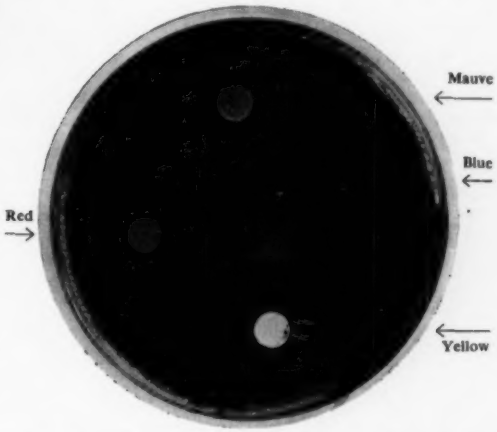

FIG. 2.—*Bact. coli* tested by the disc technique against sulphathiazole (a yellow disc), sulphafurazole (a mauve disc), "elkosin" (a red disc) and sulphadimidine (a blue disc).

TABLE II

RESULTS BY THE DRIED DISC TECHNIQUE

Organism	Number Tested	Result	Sulphathiazole		Sulphadimidine		Gantrisin		Elkosin	
			No. Sensitive	Total (%)	No. Sensitive	Total (%)	No. Sensitive	Total (%)	No. Sensitive	Total (%)
Staph. aureus ..	35	++	25	80	15	77	24	80	20	74
		+	3		12		4		6	
Strept. haemolyticus (Group A)	10	++	0	60	0	40	0	60	0	40
		+	6		4		6		4	
Pneumococcus ..	4	++	4	100	4	100	4	100	4	100
		+	0		0		0		0	
Coliforms ..	43	++	33	86	4	84	28	84	31	86
		+	4		32		8		6	
Ps. pyocyanea ..	12	++	6	67	0	0	0	17	0	25
		+	2		0		2		3	
Proteus	16	++	14	94	9	88	12	88	12	88
		+	1		5		2		2	
Total ..	120									

++ indicates that the zone of inhibition exceeded 25 mm. in diameter and + that it equalled 10 to 25 mm. in diameter.

sulphonamides gave similar qualitative results. Sulphathiazole was the most potent agent by these tests, but all compounds exhibited a wide range of activity. The quantitative differences in the zones of inhibition are of doubtful value in assessing therapeutic activity, as the *in vivo* action of the sulphonamides is influenced by many factors which cannot be controlled in the *in vitro* tests.

Discussion

These results indicate that the dried disc technique provides a useful routine method for determining the sensitivity of organisms to the sulphonamides. The technique is relatively simple and allows several sulphonamides to be tested simultaneously on one plate; a further advantage is that the technique is similar to that frequently used for the routine testing of the antibiotics. It is, however, a laboratory test and merely gives an indication of the activity of the sulphonamides under strictly *in vitro* conditions. It is well recognized that the clinical response does not always run parallel with the laboratory tests and, in the selection of a sulphonamide for clinical purposes, such factors as solubility, absorption, excretion, toxicity, and degree of acetylation and binding to the plasma proteins, must also be considered. The sulphonamides are primarily bacteriostatic agents and, in therapy, it is essential that they should reach the focus of infection in an active form and that an effective level should be maintained for some time.

Nevertheless, while sensitivity tests may provide limited information about the suitability of a sulphonamide for clinical purposes, they give a useful indication of the potential activity of the agent.

Summary

A one-stage dried disc technique for determining sensitivity to the sulphonamides has been described. This has given results comparable with those of other agar-diffusion methods.

We wish to thank Messrs. Evans Medical Supplies Ltd. for providing the sulphonamide tablets.

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J. clin. Path. (1954), 7, 73.

WASSERMANN REACTIONS SIMPLIFIED

BY

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with the technical assistance of

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(RECEIVED FOR PUBLICATION SEPTEMBER 2, 1953)

Most techniques at present in use for the Wassermann reaction require a great deal of bench and water bath space, are very time consuming, and put a considerable burden on those responsible for the preparation of glassware. The use of moulded perspex plates (M.R.C. pattern)* in this laboratory has cut down immensely the space and labour involved in doing routine Wassermann reactions, and has added considerably to the pleasure in their performance. The plates are easy to handle, no racks are required and each plate, equivalent to 80 test tubes, is washed in a few seconds. This note is submitted in the hope that others may be persuaded to review their present techniques and to consider changing or adapting them for use with perspex.

The M.R.C. pattern perspex plates, which were originally designed for virus haemagglutination tests, consist of moulded sheets of perspex 7 × 5.7 × 0.55 in. in which there are 80 vertical-sided, round-bottom cavities 0.6 in. diameter. The cavities hold about 1.5 ml. when full; a convenient working final volume is, however, 0.4 to 1.0 ml. The plates are at their best when a method is used in which reagents are added by drops. They may, however, be used with almost equal convenience with any Wassermann reaction technique in which the final volume is not more than 1 ml. Apart from any adjustment of volumes used, it is only necessary to determine by trial the periods to be allowed for fixation of complement and for lysis of red cells in order to obtain results with the perspex plates identical with those obtained in test tubes.

The technique in use in this laboratory is adapted from those described by Wyler (1929) and Orpwood Price (1949). The following is an outline of the method which has proved consistently satisfactory with the perspex plates.

Method

All sera are first put through a one "tube" screen test and at the same time in one of the plates a titration is set up of a positive serum of known titre as a control. Sera giving a positive result in the screen test are then titrated in doubling dilutions beginning at 1 in 4 with a control tube at the first dilution (i.e., 1 in 4) containing saline instead of antigen.

Reagents.—All reagents are distributed by means of dropping pipettes, the neat sera with a hand pipette delivering a small drop = 0.02 ml.; cerebrospinal fluids and dilutions of sera in quantitative tests are distributed with a hand pipette delivering a large drop = 0.10 ml.; other reagents with a fixed vertical dropper with a reservoir, delivering a large drop.

QUANTITIES OF REAGENTS

Screen W.R.s	Serum (neat) ..	1 small drop
	Antigen ..	1 large ,,
	Saline	1 ,, ,,
	Complement ..	1 ,, ,,
Quantitative Wassermann reactions and gonococcal complement fixation tests	Serum dilution ..	1 large drop
	Complement ..	1 ,, ,,
	Antigen ..	1 ,, ,,
C.S.F. Wassermann reactions ..	C.S.F. ..	1 ,, ,,
	Complement ..	1 ,, ,,
	Antigen ..	1 ,, ,,
Gonococcal complement fixation screen tests	Serum ..	5 small drops*
	Complement ..	1 large drop
	Antigen ..	1 ,, ,,

1 large drop 2·5% sensitized sheep cells added after incubation.
* This saves changing pipettes if sera for G.C.F.T. and W.R.s are put out at the same time.

Complement.—Complement is titrated according to the method of Orpwood Price (1949) and is so diluted that 1 large drop contains 1.25 M.H.D. for the test and 1 M.H.D. for controls without antigen.

Antigens.—Antigens are those supplied by the Venereal Diseases Reference Laboratory. Each batch of antigen before being taken into use is titrated chessboard fashion (Orpwood Price, 1950) to determine its optimal dilution. In our hands this is in the region of 1 in 200 for Wassermann antigen at which dilution the error using the same sized dropper for antigen and other reagents is negligible.

Period of Fixation.—We have found that incubating the plates in 37° C. incubator for 60 min. gives the same results as the 30 min. at bench temperature, followed by 30 min. in a 37° C. water-bath usually employed when glass tubes are used.

Period of Haemolysis.—After the addition of sensitized cells plates are incubated for one hour in a 37° C. incubator and the results then read (see below and illustration).

* May be obtained from Messrs. R. B. Turner & Co., Ltd., 9–11 Eagle Street, Holborn, W.C.1.

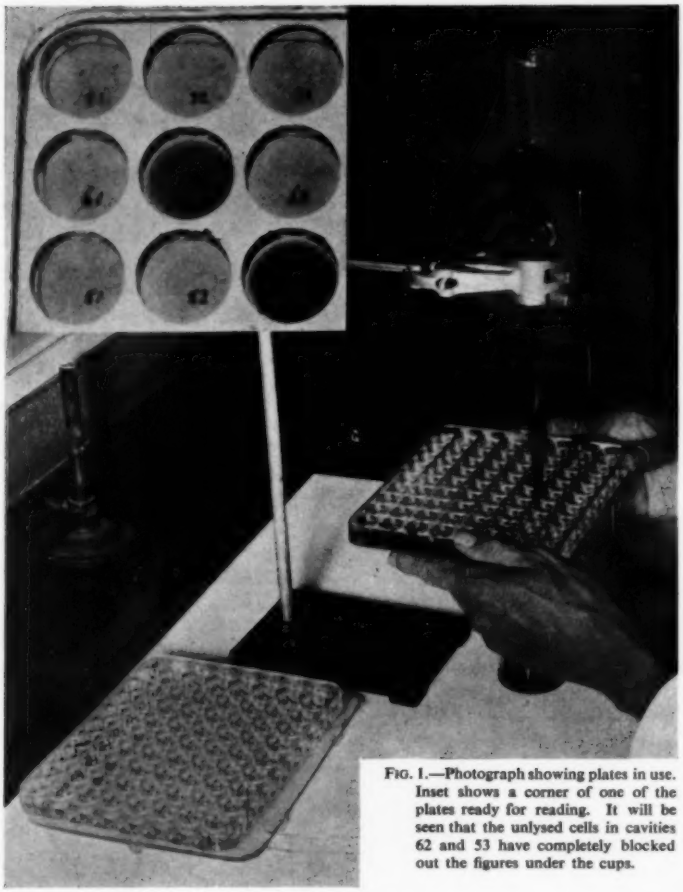

FIG. 1.—Photograph showing plates in use. Inset shows a corner of one of the plates ready for reading. It will be seen that the unlysed cells in cavities 62 and 53 have completely blocked out the figures under the cups.

Use of Perspex Plates.—It adds enormously to the convenience of using these plates if a numbered sheet of plastic or cardboard is placed underneath while sera are being put out and when results are read. The figures on the card are so spaced that one is visible under each cup in the plate. It is advisable to mark one corner of the plate so that it may be correctly orientated on the numbered sheet. It will be seen from the illustration that the numbers make the putting out of sera very simple and the reading of tests almost fool-proof, since the number under a positive cup is obscured by either the suspended or settled unlysed cells. A reasonably accurate estimate of the amount of haemolysis in any cup can be made if the cells are left to settle completely before tests are read.

Sheets of perspex cut to the size of the plates make suitable dust covers during the test and periods of incubation. It is advisable to incubate the plates in stacks of not more than two in order to avoid delay in reaching incubator temperature, although, since 75 tests (and control titration) may be done in one plate, the average batch of Wassermann reactions will seldom require more than two plates.

After use plates are washed in tap water, rinsed in distilled water, and kept in 2% HCl. If washing is not unduly delayed there is no difficulty in removing all traces of reagents.

We have performed some 3,000 tests in duplicate with plates and glass tubes and in no case has there been a difference of more than half a tube and in almost all results have been identical.

The plates are in use in this laboratory also for Paul Bunnell and Rose's tests. Results have proved consistently good.

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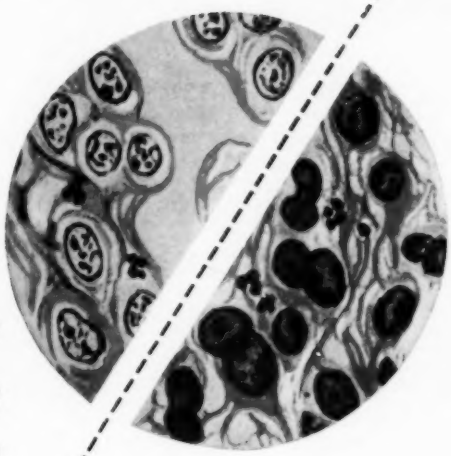

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DEMONSTRATION OF BASOPHILIC STIPPLING IN ERYTHROCYTES

BY

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From the Central Middlesex Hospital, London

(RECEIVED FOR PUBLICATION JULY 2, 1953)

The recognition of basophilic stippling in erythrocytes depends largely on subjective factors such as visual acuity and experience. An expert may observe stippling in the ordinary blood film stained with B.D.H. Leishman stain diluted with water at *p*H 6.8 to 7.0 when less expert observers may see nothing.

If one could increase contrast between the stippling and its background it would become easier to recognize. This increase in contrast can be achieved by dark-ground illumination. A stained particle under dark-ground illumination reflects that light which would be absorbed were it to pass through the stain ; it therefore appears to emit light of a colour complementary to that which it possesses by transmitted light. The thionins and thionols which constitute the basic components of all Romanowsky stains in their blue form absorb orange light, so that particles stained with them appear orange under dark-ground illumination. The red cell, if properly stained, has a pale, yellowish-green outline, within which may occur the orange-coloured dots of light which represent basophilic stippling : these particles are easily differentiated from other particles and debris.

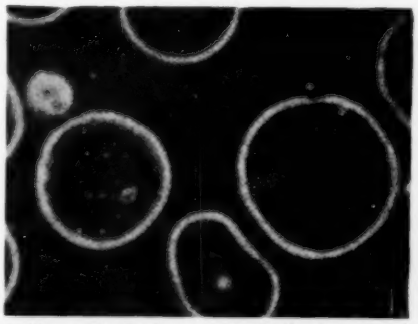

FIG. 1.—Coarse stippling in one, fine stippling in a second cell, ×2,700.

These observations are not original. Saar (1909) appears to have been the first to demonstrate dark-ground illumination of punctate basophilia in stained films, while Dietrich (1910) repeated his work and claimed that intra-erythrocytic objects corresponding to punctate basophilia could be seen in wet preparations. Ferrata and Boselli (1910) showed that, whereas the substantia reticulo filamentosa could be demonstrated only by supravital staining with brilliant cresyl blue, punctate basophilia could be demonstrated either by supravital staining or by staining a dry fixed film with methylene blue. Naegeli (1923 and 1931) states that he demonstrated punctate "basophilia" in fresh wet, stained, and unstained dry preparations by using dark-ground illumination.

Dark-ground illumination seems never to have been used for routine surveys of blood films for punctate basophilia. In my hands it is much the most certain and reliable technique, while if care is exercised in the selection of microscopic equipment it is no more troublesome than examination by transmitted light.

The film, rather heavily stained as indicated above, is examined with the optical system : focusing dark-ground illuminator, objective 1/7 in. fluorite N.A. 0.95, eyepiece ×15 straight, ×10 for inclined body tube. Illumination is with a 40 watt high-intensity filament lamp with lamp condenser throwing a slightly convergent beam. (All equipment used was by Cooke, Troughton, and Simms.) Photographs were on Kodak O 250 plates, exposure four minutes, image distance 10 in. from eyepiece, development three minutes in Kodak D 163.

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A TECHNIQUE FOR THE MEASUREMENT OF ERYTHROCYTE DIAMETERS

BY

G. C. ADAMS

From the Midwifery Department, University of Aberdeen

(RECEIVED FOR PUBLICATION JUNE 15, 1953)

The methods available for the measurement of red blood cell diameters can be classified under the following main headings: (1) The Price-Jones technique and its modifications; (2) the use of an ocular micrometer; (3) diffraction methods (Pijper, 1947); (4) a photographic method of Ponder and Millar (1924) in which the erythrocytes are photographed in plasma, unstained, and the measurement of the cell diameter made directly off the negative. The choice of any one of these methods is a personal one and will depend upon the type of work to be undertaken and the facilities available. Ponder and Millar (1924) state that erythrocytes measured by any of the methods using stained preparations are smaller due to shrinkage and that the difference in size of the cells is about 1 μ. For clinical purposes, however, and for laboratory diagnosis it is more practicable to use a method requiring a stained preparation, and of these the technique of Price-Jones (1933) is the most accurate although it is time-consuming and tedious.

In this technique, the microscope is set up in the horizontal position with the mirror removed and a point of light directed through the condenser. A prism fitted over the eyepiece of the microscope projects the image down on to the bench.

The combination of objective size, eyepiece magnification, tube length, and distance above the bench should be such that a magnification of $\times 1{,}000$ is obtained. This magnification is calibrated by the use of a stage micrometer, where the value is calculated in mm. Therefore, at a magnification of $\times 1{,}000$, 1 μ is equal to 1 mm. on the projected image. The stage micrometer is then removed and a stained blood film is placed in the stage of the microscope and the images of the cells projected on to a white paper on the bench. The outlines of the cells thus projected are drawn with a pencil and the diameters measured to the nearest 0.5 mm. The diameters of 500 cells are measured and the Price-Jones curve plotted (Whitby and Britton, 1950).

The technique described in this paper is a modification of the Price-Jones method.

Technique

Blood films are prepared by the orthodox method, care being taken to produce thin, evenly spread films for subsequent staining. Leishman stain is used. The slide is flooded with stain and left for two minutes, after which twice the volume of distilled water is added and mixed well with the stain. This is left for five to seven minutes. Leishman stain is sensitive to any change in *p*H, so that the water used in the preparation of the stain and the dilution must have a *p*H value of between 6.6 to 6.8. This is done by adding 2 ml. of a M-5 KH_2PO_4–NaOH buffer solution to each 100 ml. of distilled water used (Dacie, 1950).

After staining, the blood film is washed in buffered water until it assumes a bright rose-pink colour. It is then dried and the patient's name written on the actual film with a lead pencil. In practice it has been found that the tap water in this laboratory is of satisfactory *p*H value, and this is used in the preparation and dilution of the stain and final washing.

The apparatus is set up as illustrated (Fig. 1) in a darkened room illuminated only by a photographic safelight (yellow), and using a stage micrometer in the manner described; the magnification is adjusted to $\times 1{,}000$. With the microscope lamp still on, the stage micrometer is replaced by a stained blood film and a sheet of bromide foil card (*Kodak Data Book of Applied Photography*, 1953) is placed on the bench in the position normally occupied by the sheet of white paper in the original Price-Jones technique.

The image of the projected erythrocytes is focused critically on the foil card and the microscope lamp is switched off. The foil card used for focusing is removed and replaced with an unexposed sheet of foil card. The microscope lamp is switched on again for 30 to 40 seconds, then extinguished once more; this represents a photographic exposure, and a record of the red cell images has been made on the sensitive emulsion of the bromide foil card. The length of the exposure time must be obtained by experiment, but it has been found that using a lamp of the 12-volt "pointolite" type the given times are sufficient.

Bromide foil card is a photographic, sensitive emulsion on an aluminium base used when dimensional stability is required in photographic recording. The

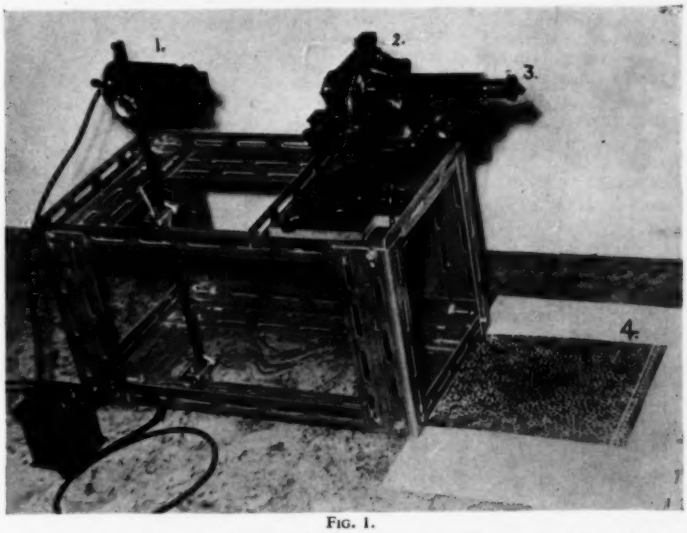

FIG. 1.—The apparatus used in this method. 1, Microscope lamp. 2, Blood film in microscope stage. 3, Drawing prism fitted over eyepiece. 4, Bromide foil card on which are projected erythrocyte images.

FIG. 2.—A typical bromide foil card recording of the erythrocyte shapes.

FIG. 3.—The glass scale for measuring diameters calibrated in mm.

aluminium base of this material enables it to lie quite flat on the bench during exposure of the projected images, ensuring critical focus to be maintained. Also, it is not liable to shrink during subsequent processing and washing, causing distortion of the shapes of the red cells.

The foil card is then developed in a metol hydroquinone developer to finality at 65° F. As the recorded image is a negative one, the red cells appear white against a black background, and when development is completed the card is fixed in a hyposulphite bath, washed and dried, when it can be taken into normal lighting conditions and the cell diameters measured when convenient.

Fig. 2 shows an example of the record made in this way.

FIG. 2.

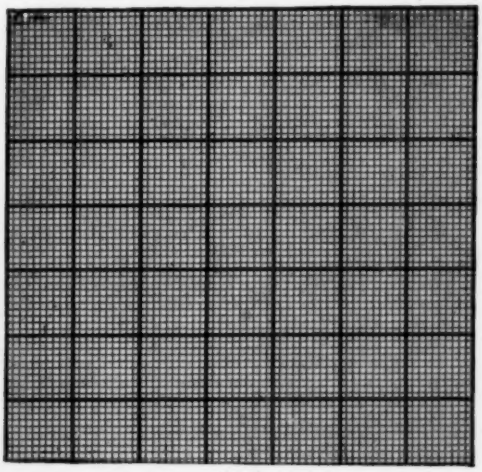

FIG. 3.

Measurements of the Red Cell Diameters

The cell diameters are measured using a glass scale calibrated in millimetres (Fig. 3) which is placed over the cell and two readings taken at right angles, thus obtaining a mean diameter. The size of the cell diameter when measured is written inside the image of the cell on the foil card. The diameters of 500 cells, from each of three fields of the stained film, are measured and the Price-Jones curve plotted (Turnbull, unpublished data).

Comment

This technique improves on the accuracy of the original Price-Jones method because actual permanent records are made of the erythrocyte shapes, which can be checked by more than one operator. A large number of preparations can be accumulated and recorded in this way during one darkroom session, the actual measurement of the cells being left until convenient, thus eliminating the tedious process of outlining the cell images in pencil in a subdued light. The foil card records can be stored with the patient's case notes or filed with research material to be referred to at any time. Also this technique does not involve the use of special equipment other than that found in most laboratories, except for the small items of photographic equipment required to process the bromide foil cards, and a room which is capable of being darkened. The time saved by this technique is considerable, as dozens of fields can be recorded in the time formerly taken to outline 500 cells by the original method.

Summary

A simple method of obtaining permanent records of the shape of erythrocytes in stained preparations for subsequent measurement of their diameters is described.

I have to thank Professor Dugald Baird for permission to publish this paper, also Dr. E. Turnbull for the use of unpublished material, and Mr. R. G. M. Drummond for photographs of the apparatus.

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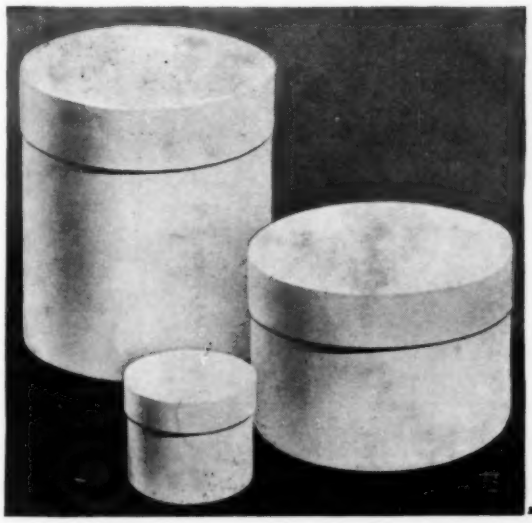

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THE QUANTITATIVE DETERMINATION OF AMINO-ACIDS IN URINE USED IN CONJUNCTION WITH ONE-DIMENSIONAL PAPER CHROMATOGRAPHY

BY

D. C. SMITH AND S. L. TOMPSETT

From the Biochemical Laboratory, Northern General Hospital, Edinburgh

(RECEIVED FOR PUBLICATION AUGUST 26, 1953)

The value of paper chromatography in the study of the amino-acids of urine in disease has been shown in particular by Dent (1946, 1948). Although present methods of one- or two-dimensional paper chromatography can indicate important quantitative changes to the observer, there is some difficulty in conveying impressions to another, e.g., to a clinician or to a reader of a paper. It does appear that a reasonably simple quantitative approach to the subject would be an advantage so that the progress of a case of variable rates of excretion by, say, the right and left kidneys, can be represented readily on paper. The following technique appears in part to fulfil this function and has been used in this laboratory for some months.

One-dimensional paper chromatography has been used. Although there is overlapping of certain of the amino-acids, it has been proved more convenient for day-to-day observations in the clinical laboratory. More specific information can naturally be obtained if necessary by the use of two-dimensional paper chromatography. "Runs" are made in the "kawerau" unit (1951). This has proved of great convenience since an incubator can house several of these units, a constant temperature being thereby maintained. The solvent used is n-butanol/acetic acid. Amino-acid nitrogen is determined quantitatively by a modification of the colorimetric ninhydrin procedure of Smith and Agiza (1951). The following represents the main departure from the usual procedure. Instead of locating individual amino-acids by their R_F values the paper is divided into 10 strips corresponding to R_F 0.0–0.1, 0.1–0.2 . . . . . 0.8–0.9, 0.9–1.0 and the amino-acid nitrogen content of each strip determined. The results are then plotted in the form of a graph, mg. amino-nitrogen per 100 ml. against R_F. This allows of ready comparison. Undoubtedly some amino-acids will make their appearance on adjacent strips.

Procedure

Apparatus.—The following is required.

(1) Kawerau unit (Quickfit and Quartz Ltd.) maintained in an incubator at 37° C.

For the solvent, 40 ml. of n-butanol, 10 ml. of glacial acetic acid, and 50 ml. of distilled water are shaken in a separating funnel. The non-aqueous phase is placed in one flask and the aqueous phase in the other. Since some esterification may occur with a consequent change in R_F, frequent changes of the solvent are advisable.

(2) Shandon micro-capillary pipettes.

(3) Whatman filter paper, No. 1, in strips of 20 mm. width.

(4) Unicam spectrophotometer, G.P. 350.

Reagents.—The following should be made up:

Citrate Buffer pH 5.—Citric acid, 21.008 g., is dissolved in 200 ml. of water ($C_6H_8O_7.H_2O$), 200 ml. of N sodium hydroxide added and diluted to 500 ml. with water.

Ninhydrin Reagent.—Ninhydrin, 550 mg., is dissolved in 100 ml. of citrate buffer.

Stannous Chloride Reagent.—Stannous chloride, 0.1 g., is dissolved in 50 ml. of citrate buffer. This reagent must be prepared freshly.

Technique.—Urine, 20 µl., measured by means of a "shandon" micro-capillary pipette, is placed just in front of a pencil line drawn across the filter paper strip. After drying the paper is arranged in the apparatus so that the end nearest the pencil line dips into the organic solvent mixture. The apparatus is placed in an incubator at 37° C. for 24 hours. At the end of this period the paper is removed, the position of the solvent front marked with a pencil line, and then allowed to dry at room temperature.

The paper between the two pencil marks (approximate length 30 cm.) is divided into 10 equal parts, corresponding to R_F ranges 0.0–0.1, 0.1–0.2 - - - - - - - - 0.9–1.0. Each strip is placed in a separate test-tube and 5 ml. of water, 2 ml. citrate buffer, 2 ml. nin-

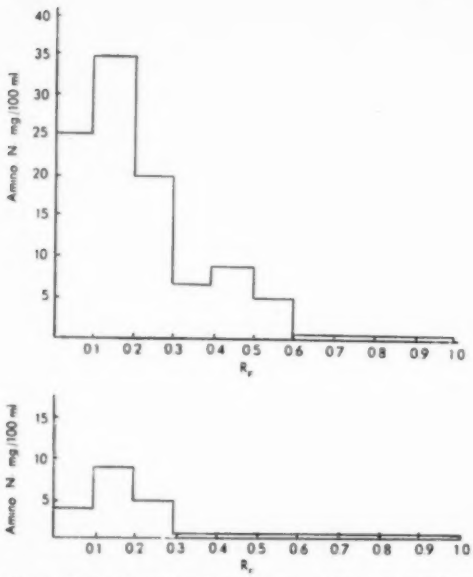

FIG. 1.—Graph of a case of liver damage. Above: on admission, amino-N was 96 mg./100 ml., and on discharge (below) it was 14.5 mg./100 ml.

hydrin reagent, and 1 ml. stannous chloride reagent added in order.

The tubes are placed in a boiling water-bath for 15 minutes and then cooled in cold water in the dark. The absorption of the purple colour which results is then measured in any suitable absorptiometer at 570 mμ. against tube No. 10 (R_F 0.9–1.0).

Results are read off a graph prepared from a standard solution of glycine (1–5 μg. amino N/5 ml.).

The positions occupied by the various amino-acids on the strips can be determined by submitting solutions of the pure substances to the above treatment.

The attached figure represents the method of recording the results. These are from a case of liver disease on admission and on discharge.

Discussion

Tube No. 10 has been used as the blank since none of the known amino-acids have R_F values approaching this value when the above solvent system is used.

Equivalent amounts of different amino-acids do not give the same depth of colour with the ninhydrin reaction, the depths varying from 100% for glycine, leucine, iso-leucine, and nor-leucine to 79% for cystine (Smith and Agiza, 1951). Corrections have not been applied, since, in this type of examination, it would be most difficult, and, in any case, clinical interpretations would hardly be affected.

When warranted, it has been possible to apply more specific colour reactions to the strips suspended in water for the quantitative determination of certain amino-acids, e.g., the Pauly reaction (histidine, histamine), the phenol reagent (tyrosine, tyramine). Although the phenol reagent itself is non-specific in the above solvent system, the usual urinary phenols have R_F values much higher than tyrosine or tyramine.

Summary

A procedure for the quantitative determination of amino-acids in urine by means of the ninhydrin reaction after one-dimensional paper chromatography has been described.

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THE ESTIMATION OF TOTAL NITROGEN USING THE CONWAY MICRO-DIFFUSION CELL

BY

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From the Wellcome Research Laboratories, Biological Division, Beckenham, Kent

(RECEIVED FOR PUBLICATION JUNE 3, 1953)

The use of the microdiffusion unit for the determination of blood urea has been described by Conway and O'Malley (1942). Recovery of urea added to blood when estimated by this method is precisely quantitative as compared with a 98 to 99% recovery given by the aeration method (Kay and Sheehan, 1934). The method has been found very useful for routine laboratory determinations, especially when dealing with large numbers of samples.

Conway (1939a) has also described a method for determining total nitrogen, using the diffusion cell to estimate the ammonia after digestion by the usual Kjeldahl technique. A modification of the method so that the same reagents as those used for blood urea estimation can be employed is described. The method is of considerable value, especially when only small amounts of sample are available or large numbers of estimations have to be performed, since distillation is avoided and all titrations may be carried out together, if necessary on the day after setting up the units.

Experimental

Reagents.—The following are required:

Potassium sulphate, analytical reagent.

Sulphuric acid, concentrated, analytical reagent.

Copper sulphate, saturated solution.

Selenium dioxide, 0.06% (w/v) aqueous solution.

Sodium hydroxide, 40% (w/v) aqueous solution.

Potassium metaborate, saturated aqueous solution.

Tashiro's Indicator.—Methylene blue solution in alcohol, 5.0 ml. of 0.1% (w/v), is added to 200 ml. of 0.1% (w/v) alcoholic solution in methyl red.

Boric Acid Absorbent.—Boric acid, 5.0 g., is dissolved in distilled water, 200 ml. absolute alcohol and 10 ml. Tashiro's indicator added, and the volume adjusted to 1 l. with distilled water.

Hydrochloric acid, 0.02 normal.

Digestion.—For quantities up to 0.2 ml. the sample is digested in a small Kjeldahl flask prepared from a 12.5×1.5 cm. test-tube with a bulb, 2.5 cm. in diameter, blown in the bottom. The sample is mixed with 0.5 g. potassium sulphate, 0.5 ml. concentrated sulphuric acid, and 0.03 ml. copper sulphate. The mixture is heated gently for five minutes, allowed to cool somewhat, and 0.3 ml. selenium dioxide added. The heating is then continued so that the mixture boils gently for one hour, care being taken, especially in the early stages, to avoid loss by spitting. If these conditions are carefully observed digestion is complete in one hour. After cooling, the mixture is washed into a 10-ml. volumetric flask with distilled water, 0.375 ml. sodium hydroxide added to neutralize the bulk of the acid, and the volume adjusted to 10 ml.

For quantities of 0.5 ml. or more, the digestion is carried out in a 50-ml. Kjeldahl incinerating flask and 10 times the amounts of reagents are used. The mixture is diluted in a 50-ml. volumetric flask, using 3.75 ml. of sodium hydroxide to neutralize most of the acid before adjusting to volume.

Estimation of the Ammonium Sulphate.—Boric acid, indicator absorbent, 2.0 ml., is placed in the centre compartment of a clean Conway diffusion unit and 0.5 ml. or 1.0 ml. of the solution for estimation carefully run into the outer compartment. The lid is thoroughly coated with white soft paraffin and placed on the dish, leaving a small aperture to allow 1.0 ml. of potassium metaborate to be introduced into the outer chamber. The aperture is closed immediately and the liquids in the outer compartment mixed by gentle rotation, care being taken not to splash the liquid from one chamber to the other. The minimum time for complete absorption of the ammonia is two hours, and titration may be carried out at any time after this period. If desired, the units may be left overnight.

The amount of ammonia absorbed is estimated by titration with 0.02 normal hydrochloric acid, the indi-

TABLE I

ESTIMATION OF THE NITROGEN CONTENT OF A SOLUTION OF UREA

Quantities			Concentration N_2 Found (%)
Volume Digested (ml.)	Adjusted Volume after Digestion (ml.)	Volume for Titration (ml.)	
Micro-method			
0·05	10·0	1·0	1·168±0·004
0·10	10·0	1·0	1·154±0·002
0·20	10·0	0·5	1·148±0·001
0·50	25·0	0·5	1·157±0·002
	Overall mean		1·157±0·002
	% recovery		99·8
Macro-method			
0·5	50·0	1·0	1·152±0·002
1·0	100·0	1·0	1·156±0·002
2·0	100·0	1·0	1·149±0·006
5·0	500·0	1·0	1·157±0·002
	Overall mean ..		1·154±0·002
	% recovery		99·5

The results are the mean of four determinations ± S.E. Urea solution is equivalent to 1·16% nitrogen.

cator change being from green to pink, and titration is continued to a faint but definite pink. An "agla" microsyringe (Trevan, 1925) which will deliver 0.001 ml. with an accuracy of ±0.00005 ml. is used for the titration. A glass tube, bent at right angles with the end drawn out to a point, is sealed on to the nozzle of the syringe. The point is allowed to dip just below the surface of the liquid during the titration and the solutions are mixed with a fine glass rod. The micrometer screw attached to the syringe may be manipulated by hand, but it is convenient to accelerate the initial stages of the titration by driving the screw by means of a low-voltage induction motor. The quantities of sample and the dilution of the product should be adjusted to give a titration figure of 0.4 ml. to 0.5 ml.

It is important that the microdiffusion units should be thoroughly clean, and the procedure described by Conway (1939b) must be carefully followed.

Results

The recoveries from a series of determinations of a solution of urea using varying quantities of sample are given in Table I. Comparison of the results with the micro- and macro-method shows that the recovery is equally satisfactory by both methods and with all the amounts of solutions used, except for the estimation using 0.05 ml. of sample, which gave a uniformly high result, although the difference is barely significant.

The results of analysis of samples of serum and urine from two dogs are shown in Tables II and III. The samples were estimated alone and after the addition of urea solution equivalent to an additional nitrogen concentration of 1.16%.

In serum the results were equally satisfactory with all quantities taken for the estimation, and overall recoveries of 100.4% and 98.9% were obtained for the two samples with standard errors for a single determination of ±0.22 and ±0.26 respectively.

There was somewhat greater variation in the individual determinations in the case of the urine estimations (Table III), the overall mean recoveries being 98.8% and 101.2% with standard errors for a single determination of ±0.34 and ±0.52.

In both serum and urine estimations satisfactory results are obtained when only 0.05 ml. and 0.5 ml. of sample respectively is used, clearly illustrating the value of the method when only small amounts of material are available.

TABLE II

ESTIMATION OF THE NITROGEN CONTENT OF SAMPLES OF SERUM AND URINE AND THE RECOVERY OF NITROGEN ADDED TO SERUM AND URINE

Quantities			Sample 1			Sample 2		
Volume Digested (ml.)	Adjusted Volume after Digestion (ml.)	Volume for Titration (ml.)	Concentration N_2 (%)	Sample+1·16% N_2		Concentration N_2 (%)	Sample+1·16% N_2	
				Total N_2 Found (%)	Added N_2 (% Recovery)		Total N_2 Found (%)	Added N_2 (% Recovery)
	Serum							
0·05	10·0	1·0	0·821±0·007	1·993±0·005	100·9	0·849±0·003	1·995±0·004	98·8
0·10	10·0	1·0	0·827±0·003	1·982±0·003	99·5	0·848±0·004	1·995±0·004	98·9
0·20	10·0	0·5	0·825±0·003	1·996±0·002	100·9	0·854±0·005	2·000±0·004	98·8
0·50	10·0	0·5	0·827±0·002	1·990±0·005	100·2	0·860±0·005	2·004±0·003	98·6
	Overall means ..		0·825±0·002	1·990±0·002	100·4	0·852±0·003	1·999±0·002	98·9
	Urine							
0·5	100·0	1·0	0·723±0·002	1·879±0·004	99·6	2·075±0·003	3·258±0·011	101·8
1·0	100·0	1·0	0·719±0·003	1·861±0·005	98·4	2·082±0·007	3·254±0·011	100·9
2·0	100·0	0·5	0·722±0·002	1·864±0·008	98·6	2·087±0·005	3·270±0·007	101·8
5·0	500·0	0·5	0·721±0·001	1·866±0·012	98·6	2·088±0·013	3·251±0·008	100·2
	Overall means ..		0·721±0·001	1·868±0·004	98·2	2·083±0·003	3·258±0·004	101·2

The results are the mean of four determinations ± S.E.

TABLE III

ESTIMATION OF URINE NITROGEN AND RECOVERY OF NITROGEN ADDED TO URINE

Quantities Taken	Dog 1			Dog 2		
	Urine Alone	Urine+Urea=1·16% Added Nitrogen		Urine Alone	Urine+Urea=1·16% Added Nitrogen	
	Nitrogen (%)	Total Nitrogen (%)	Added Nitrogen (% Recovery)	Nitrogen (%)	Total (Nitrogen %)	Added Nitrogen (% Recovery)
0·5 ml.→100 ml.	0·722	1·874	99·3	2·076	3·268	102·5
1·0 ml. for titration	0·721	1·876	99·5	2·070	3·244	101·1
	0·719	1·892	101·2	2·080	3·284	103·7
	0·728	1·874	98·8	2·068	3·236	100·6
Mean±S.E. ..	0·723±0·002	1·879±0·004	99·6±0·22	2·075±0·003	3·258±0·011	101·8±0·29
	0·722	1·864	98·4	2·088	3·264	101·2
1·0 ml.→100 ml.	0·721	1·870	99·1	2·062	3·280	104·9
1·0 ml. for titration	0·711	1·860	99·1	2·086	3·236	99·1
	0·723	1·848	97·0	2·090	3·236	98·8
Mean±S.E.	0·719±0·003	1·861±0·005	98·4±0·20	2·082±0·007	3·254±0·011	100·9±1·55
	0·724	1·870	98·8	2·100	3·260	100
2·0 ml.→100 ml.	0·720	1·882	100·1	2·088	3·260	99·3
0·5 ml. for titration	0·721	1·842	96·7	2·084	3·288	103·6
	0·726	1·862	97·9	2·074	3·272	103·1
Mean±S.E.	0·722±0·002	1·864±0·008	98·6±0·08	2·087±0·005	3·270±0·007	101·8±1·22
	0·722	1·868	98·8	2·061	3·256	102·8
5·0 ml.→500 ml.	0·722	1·878	99·6	2·100	3·236	97·9
0·5 ml. for titration	0·720	1·832	95·8	2·073	3·268	102·8
	0·722	1·884	100·1	2·075	3·252	101·2
Mean±S.E.	0·721±0·001	1·866±0·012	98·6±1·26	2·088±0·013	3·251±0·008	100·2±1·68
Overall mean±S.E.. .	0·721±0·001	1·868±0·004	98·8±0·34	2·083±0·003	3·258±0·041	101·2±0·52

Summary

The value of the Conway microdiffusion method for a series of total nitrogen estimations is discussed.

A method using the same reagents as those required for blood urea determinations is described.

The results of estimations with urea solution, serum and urine, using varying amounts of sample, are considered.

The method is particularly useful when only small amounts of material are available, since satisfactory results may be obtained using only 0.05 ml. of sample.

I wish to thank Miss A. Facey and Miss M. Green for valuable technical assistance.

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J. clin. Path. (1954), 7, 84.

A BRANDING IRON FOR MARKING HISTOLOGY TISSUES

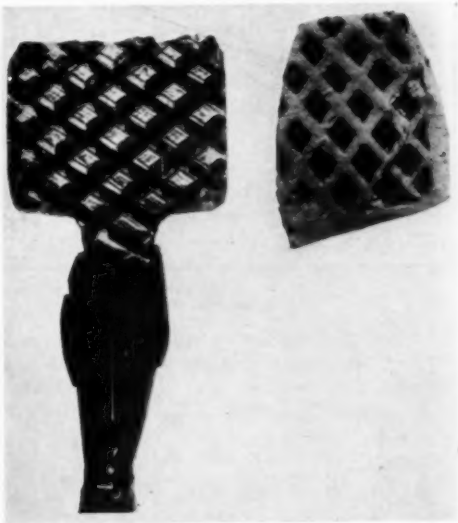
BY

I. M. TUCK

From the North-Western Group Laboratory, London

(RECEIVED FOR PUBLICATION MARCH 30, 1953)

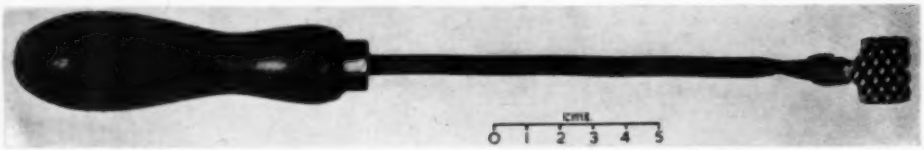
Every histologist experiences from time to time the annoyance of having sections cut from the wrong face of a piece of tissue. Even the time-honoured method of marking the surface to be blocked uppermost by shaving off the edge of one side occasionally fails, especially with the thinner slices that tend to be cut nowadays for the automatic tissue processors. The branding iron here described is designed to overcome this difficulty.



The head consists of a block of copper, 2 × 1.5 cm. and 0.75 cm. thick, with a tapered tongue 2 cm. long projecting from the centre of one of the longer sides. On one surface diagonal cuts in opposite directions are made with a hacksaw and these are further opened with the edge of a half round file, so leaving a pattern of diamond-shaped projections.

The tapered tongue is riveted into the split end of a piece of steel rod which has a wooden handle at the other extremity.

In use the head is heated in a bunsen flame to a dull red and the patterned surface momentarily applied to the face of the tissue opposite to that from which sections are to be cut. The period of contact is so short that the specimen suffers no harm ; the marking is distinct and is unaltered by subsequent processing.



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BOOK REVIEWS

Traitement des Hémorragies. By Jean-Pierre Soulier. (Pp. 217. Fr. Frs. 1,400.) Paris: Les Editions Médicales Flammarion. 1953.

In the first section of this book, after a brief reference to the methods of investigating haemostatic function, the author deals soundly and critically with the various drugs and other preparations for which a haemostatic action has been claimed, beginning with adrenalin and ending with the transfusion of concentrated platelet suspensions.

The second part of the book is concerned with the treatment of the haemorrhagic syndromes one by one. Here Dr. Soulier's recommendations are generally in line with current British practice. For example, in a discussion on the treatment of haemorrhage in haemophilia the need for large and repeated transfusions is stressed; 300 to 500 ml. of fresh blood or plasma are regarded as the minimum volumes likely to result in any appreciable benefit in the adult. Transfusions given at intervals in order to forestall haemorrhage are not recommended because of their transient effect and because of the possible danger of the formation of circulating anticoagulants. Splenectomy is recommended for all patients with idiopathic thrombocytopenic purpura whose disease has lasted more than one year and who suffer from frequent haemorrhages.

The third part of the book deals with the treatment of haemorrhage from various sites and organs. This section, which necessarily deals with such subjects as the treatment of uterine fibroids and a discussion on surgical intervention for gastric haemorrhage, will be of less interest to the haematologist.

In all, there are 193 pages of text, and of the 98 references in the bibliography it is noteworthy that more than half of them have been taken from British or American sources.

J. V. DACIE.

Disorders of the Blood. By Sir Lionel Whitby and C. J. C. Britton, 7th edition. (Pp. xii+856, 20 plates, 106 figures. 63s.) London: J. & A. Churchill. 1953.

As the authors point out in the preface to the new edition of this standard English textbook, the task of maintaining a textbook on a rapidly growing subject is difficult, but the authors have managed to put into this new edition several chapters which indicate to the general reader the advancing line of haematology in all its aspects. This is particularly noticeable in the new chapters on immuno-haematology and blood coagulation. The new British work on coagulation is here given prominence, and the very adequate details of the new tests and their evaluation should give to the practising pathologist a clearer understanding of pseudo-haemophilia, Christmas disease, and other coagulation defects. It is rewarding to read the clear descriptions of such subjects as the transport of iron, haemoglobin synthesis, the place of B_{12} in haemopoiesis as well as in treatment, and a clarification of the haemolytic anaemias by the use of the variety of tests for haemolysins and the survival times of transfused red cells. On the subject of immunology the authors take a classic stand, and an excellent chapter on the prevention and treatment of transfusion reactions is particularly noteworthy.

The book ends, as previously, with a section on practical methods, and here again the previous standard is maintained with brevity and clarity of technical descriptions which are easy to follow in every case. It is certainly gratifying to see this standard textbook maintaining and even improving upon its previous high standard and brought well up to date with mention in the text and in the references of the most recent accepted advances in haematology. Unfortunately the increase in the size of the subject and therefore of the book from 759 to 856 pages has also meant an increase in price.

A. GORDON SIGNY.

The Megaloblastic Anaemias. By L. J. Davis and A. Brown. (Pp. xi+113; 12 figures. 21s.) Oxford: Blackwell Scientific Publications. 1953.

This eminently readable small monograph has been written for the general physician rather than for practising haematologists. Even so it is noteworthy for an admirable presentation of a difficult subject still full of controversy, with a style which makes the clinical and pathological aspects readily understood. The management of patients with megaloblastic anaemias is outlined in some detail and the importance is stressed of the laboratory investigations before treatment is started and adequate laboratory control in the follow-up. The seven photomicrographs show a uniform excellence and demonstrate normal and abnormal erythropoiesis. Two hundred and seventy-eight references are included, but it must be said that the price appears excessive for a monograph of this size.

A. GORDON SIGNY.

Nature and Structure of Collagen. Edited by J. T. Randall, assisted by Sylvia Fitton Jackson. (Pp. 269; illustrated. 42s.) London: Butterworths Scientific Publications. 1953.

A discussion was convened by the Colloid and Biophysics Committee of the Faraday Society at King's College, London, in March, 1953. Twenty-four papers were presented. These have now been published, together with verbatim reports of the discussions which they aroused.

Owing to the participation of experts from almost every branch of science concerned with fibre structure and fibrogenesis, the volume presents a remarkably comprehensive yet compact account of the present state of knowledge in this extremely important field. Subjects

dealt with at varying length include histology, histo- and micro-chemistry, collagenase, collagen metabolism, relationship of reticulin to collagen, *x*-ray crystallography, electron microscopy, antigenicity, pro-collagen and connective tissue polysaccharides.

The increasing use of verbatim reporting of scientific discussions is to be deplored. Much space could be saved and greater clarity of expression attained by adequate editing and conversion to reported speech. The importance of this volume to all workers interested in the "collagen diseases" is obvious. It is therefore most regrettable that two comparatively minor defects seriously affect its value as a book of reference. These are the absence of an author index and the extraordinarily inadequate subject index. The rapidity, however, with which the book has been published may perhaps be held to account for these defects.

The quality of the contributions is uniformly high, and it would be invidious to distinguish any for special mention. The book can certainly be recommended as providing the most up-to-date and authoritative account of its subject, and it emphasizes once again how wide can be the approach to even a narrow sector of knowledge, and how essential, therefore, are those organizations designed to coordinate the workers in apparently unrelated sciences.

L. E. GLYNN.

Dr. D. N. Nabarro

We offer our congratulations to Dr. D. N. Nabarro whose eightieth birthday is on February 27, 1954.

Dr. Nabarro was a pioneer in clinical pathology as it is known today. He was much concerned with the early work on immunization, he opened the first clinic specifically for testing and treating children suffering from congenital syphilis, and in 1903, with Bruce and Castellani, he confirmed the discovery of trypanosomiasis in the C.S.F. in cases of sleeping sickness.

Dr. Nabarro is a past-president of the Association of Clinical Pathologists, and is Emeritus Director of Pathology at The Hospital for Sick Children, Great Ormond Street.

International Congress of Clinical Pathology

An international congress of clinical pathology will be held from September 6 to 11, 1954, in Washington, D.C., United States of America. There will be some concurrent and some joint meetings with the Fifth International Conference on Geographic Pathology and of the International Association of Medical Museums. In addition, the College of American Pathologists will sponsor a half-day symposium on the first day and the American Society of Clinical Pathologists will conduct the regular seminar on the day after the congress.

All meetings and the exhibits will be at the Shoreham Hotel.

The programme of the congress will include all aspects of clinical pathology, and sectional meetings will be arranged on the basis of diseases and organic systems, such as metabolic diseases, tuberculosis, disease of bone, etc., rather than by disciplines, such as chemistry, microbiology, etc. The programme of the Conference on Geographic Pathology will be devoted entirely to cancer. The meeting of the International Association of Medical Museums will provide an opportunity to discuss the teaching of pathology and the use of technical methods in teaching and research.

Monday, September 6.—Morning, joint opening session.
Afternoon, symposium of College of American Pathologists.

Tuesday, September 7.—Morning, joint scientific session of the Congress of Clinical Pathology and Conference of Geographic Pathology on the geographic distribution of cancer.
Afternoon, sectional meetings.

Wednesday, September 8.—Morning, sectional meetings.
Afternoon, free for visits to scientific institutions.
Evening, Askanazy Lecture of the International Society of Geographic Pathology.

Thursday, September 9.—Morning, sectional meetings.
Afternoon, sectional meetings.

Friday, September 10.—Morning, sectional meetings.
Afternoon, joint scientific session on the geographic distribution of diseases other than cancer.

Saturday, September 11.—Annual seminar of the American Society of Clinical Pathologists.

Ample space in the headquarters hotel will be available for exhibits, and both those who will present papers and those who will not are urged to prepare exhibits. Inquiries concerning space and facilities should be directed to Col. Hugh Gilmore, Chairman of Committee on Exhibits, Armed Forces Institute of Pathology, 7th and Independence, S.W., Washington 25, D.C., United States of America.

A comprehensive exhibit on the world-wide distribution of cancer is being prepared for the Conference of Geographic Pathology.

The official languages of the congress will be English, French, German, and Spanish. Simultaneous translation in these four languages will be available at the joint sessions and at some of the sectional meetings.

The registration fee for those attending the congress will be $25. This includes a programme with abstracts of all papers, and tickets for all receptions, and the official dinner. Registrants at the Congress of Clinical Pathology are welcome to attend sessions of the Conference of Geographic Pathology and meetings of the Association of Medical Museums without additional registration.

Anyone wishing to attend should write to Dr. Thomas M. Peery, Chairman of General Committee on Registration, Department of Pathology, George Washington University Hospital, Washington 7, D.C., United States of America.

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THE USE AND LIMITATIONS OF FILTER-PAPER ELECTROPHORESIS

BY

N. H. MARTIN AND G. T. FRANGLEN

From the Department of Chemical Pathology, St. George's Hospital, London

(RECEIVED FOR PUBLICATION APRIL 2, 1954)

In 1935 Tiselius, using the apparatus he had devised in 1930, demonstrated that human serum globulin fell into three reasonably defined groups based on their electrophoretic mobilities. For the next 15 years a steady flood of papers reported the fluctuations of albumin and the globulins in young and old, in health and disease, as disclosed by analyses carried out using Tiselius apparatus with and without the numerous minor modifications which were introduced.

In spite of brilliant efforts at simplification it was evident that the method, because of its relative complexity, could never have widespread use in routine clinical chemistry. In 1950 three groups of workers, among them Tiselius himself, described methods which indicated that, using filter paper as a matrix, a relatively cheap and rapid method could be evolved of obtaining a rough analysis of the serum proteins akin to the analysis obtained by the classical technique.

The method required no more than 0.5 ml. of serum, and though estimates of the total protein concentration would still have to be made by other means, it was conceivable that this simple technique would dispense with the tedious salt fractionation and subsequent analyses required heretofore to obtain the albumin/globulin ratio.

The last four years have produced a spate of papers devoted to the description of modifications in methods of separation and analysis with numerous technical and mathematical tricks for improving the correlation of the results with the substantial body of data already obtained with the classical technique. With few notable exceptions scant attention was paid to the fundamental principles underlying the technique or to the points in which their application to it differed from the classical procedure.

As paper succeeded paper it became increasingly evident that except within fairly wide limits the two methods were not quantitatively comparable. Even worse, because of lack of time spent on a consideration of the basic principles involved, it seemed probable that differing techniques for the production of "electrophoretograms" were not comparable among themselves.

One was faced with the prospect of scores of laboratories turning out hundreds of results year by year; each group, because of deviations in technique unappreciated and therefore unrecorded, differing to an unknown extent one from the other. The clinical chemical chaos that could arise demanded a reassessment of the situation in the hope that workers in the field would stop, think, and start again.

Electrokinetic phenomena, as the derivation of the word implies, are concerned with the production of electrical manifestations by the movements of particles in liquids and conversely the production of orientated movement of particles in a liquid medium when an electrical current is passed through it.

In 1809, barely 10 years after Volta had built the first galvanic cell, Reuss, a Russian physicist, stuck two vertical glass tubes into a piece of wet clay, filled each with water and then carefully placed a layer of fine sand in the bottom of each tube. The terminals from the battery which he had fashioned, out of 74 roubles and 74 zinc pieces, were led into the tubes. When the switch on his circuit was closed he observed that the clear water at the positive pole became milky from the migration of fine particles. Moreover, he noted that though the water at the negative pole remained clear its volume increased. This experiment is the first clearly recorded observation of two electrokinetic effects, electrophoresis, the migration of the clay particles through the water, and electro-osmosis (endosmosis), the migration of water through the porous plug of clay.

The other two electrokinetic effects which may be represented as the converse of these two observations are the production of an electrical potential by the flow of a liquid through a tube, and the production of an electrical potential by

the movement of particles sedimenting in a liquid column. The first of these was described and investigated by Quinke in 1859 and 1861. It is commonly referred to as streaming potential. The second was examined by Dorn in 1880 and is referred to as sedimentation potential or the Dorn effect.

All these four phenomena are usually attended by certain secondary effects due to the inevitable modifications of the existing properties of the system in which they take place. Perhaps the most important is the electroviscous effect which is the demonstrable increase in viscosity of the solution during the application or production of the electrical potential. In addition the dissipation of heat produced during the passage of an electrical current may materially alter the spatial relation of particles migrating under the current's influence by the creation of secondary convections.

The development of electrokinetic theory has shown that the four principal phenomena have much in common and that the laws which hold for one apply with only minor modification to all. It is evident too that in practice it is very difficult to create an apparatus for the demonstration of the electrophoretic effect that will not also involve the consideration of one of the other effects, streaming potential or electro-osmosis.

It is important to realize that though clay and water were the materials with which Reuss made his initial experiments, had he used soda water or champagne he might have observed the ordered migration of the bubbles of gas just as he did the particles of clay. Moreover, suspensions of oil droplets in water will migrate with equal facility to the positive pole though they are not normally regarded as existing in an ionized form in the sense that the sodium ion or chlorine ion would be. It would appear then that these particles have in some way acquired a charge and it was in attempts to explain how and why they could acquire a charge that the concept of the electrical double layer developed. The modern concept is that the differences in behaviour of the large and small molecules are ones of degree, and are all finally related to the distribution of electron densities in the system.

The Tiselius Apparatus

Three techniques have been developed in research laboratories for the measurement of electrophoretic movement. Of these, " the moving boundary technique," in which the movement of the boundary is measured between a solution of the molecule being investigated and the solvent, had fallen into disrepute because of the technical difficulty of forming, maintaining, and observing the boundary with sufficient accuracy.

In 1930 Tiselius published his thesis. In the next seven years, so great were the improvements in the technique initiated by him (1937, 1938) that from being the Cinderella among the methods for measuring the electrophoretic effect the moving boundary technique became one of the most popular. It was not only that these improvements enabled new light to be thrown on old problems but that it now became possible to analyse mixtures of soluble proteins and to characterize them without materially modifying their intrinsic structure. This was revolutionary, and it is proper therefore to recapitulate the improvements which Tiselius initiated.

The Construction of the U Tube.—Burton in 1906 had used a simple U tube for the study of boundary migration. Over the next 20 years various modifications had been made to it. But Tiselius altered its shape from a cylindrical to an oblong cross-section, so that not only could accurate observations be made on the deflection of parallel beams of light of any wavelength passing through the tube and the liquid it contained, but convections due to uneven heat dissipation through the liquid in the tube were greatly reduced.

The Formation of the Boundaries.—Tiselius introduced a shearing technique and obtained by it much clearer and more consistent starting boundaries than had previously been obtained by the old methods of displacement. This he did by breaking his tube into four sections: the top section, which was simply the connecting link between the U tube proper and the electrode vessels; the upper and lower middle sections, which formed the limbs of the U and through which the analytical measurements were made, and the base section which contained the linking channel between the two limbs (Figs. 1 and 2). The boundaries were made by sliding the lower of the middle sections across between the upper and the base sections after complete temperature equilibration.

The Elimination of Density Gradients.—As Tiselius was largely concerned with observations in aqueous solution, he appreciated and arranged that the solution being examined, being more dense than the buffer against which it had been dialysed, should be placed in the bottom sections of his U tube, and that equilibration and migration should be carried out at or near 4° C., the maximum density of water.

The Observation of Colourless Boundaries.—To observe the movement of the colourless boundaries with which he was largely concerned, he borrowed from the physicists the delicate Töpler–Schlierin method for observing minute changes in refraction. This method had been used for many years to test lenses for chromatic and spherical aberration. It depends on the fact that, if parallel light beams are brought to a point of focus by the lens under test and a screen placed at the focal point of the lens so that it just covers the image of the light source produced there, the field seen in a telescope placed beyond the screen and focused on the lens will appear dark provided there are no defects in the lens which have deflected the beams of light. If there are any such defects these will appear luminous in the otherwise dark field.

Töpler (1867) realized that the "defect" could be a small variation in refractive index in a column of liquid placed immediately in front of a perfect lens and used the technique for measuring minute variations of refractive index in liquids. It is from his modification of the original technique that modern Schlierin scanning techniques have all been developed.

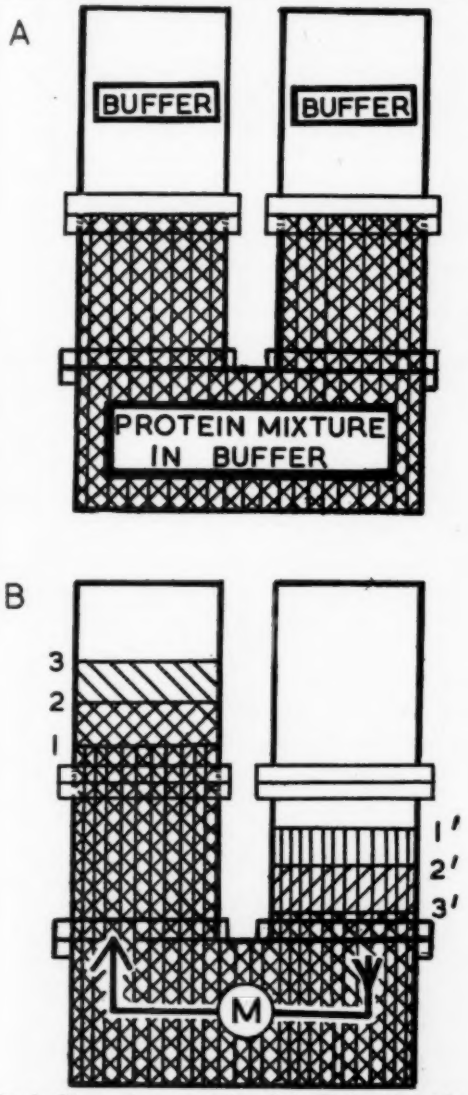

FIG. 1.—Diagramatic representation of the Tiselius "U" tube. A shows the starting boundaries. B shows the position of the boundaries after passage of the current. M shows the direction of the current. 3 2 1 show the moving boundaries on the "ascending side." 3′ 2′ 1′ show the moving boundaries on the "descending side."

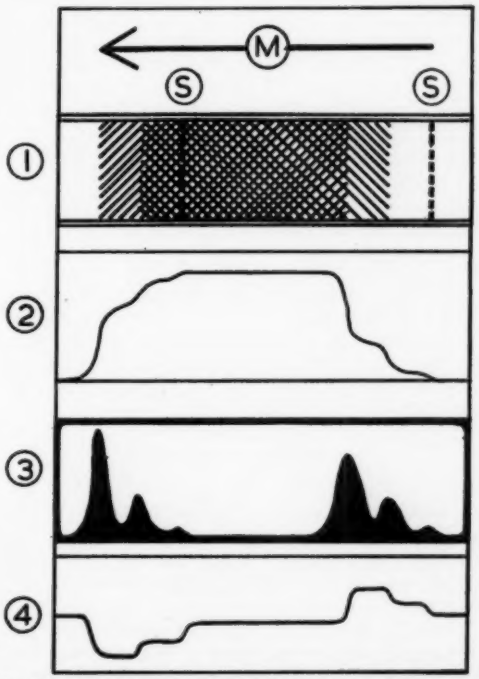

FIG. 2.—1 is a schematic representation of two proteins migrating in the classical apparatus (the U tube has been straightened). M = Direction of migration. S = Salt protein boundaries. 2 is a tracing of changes of the refractive index over the area of migration. 3 is the Schlierin pattern—the rate of change of the refractive index over the area of pattern. 4 shows the variation of conductivity through the area of migration illustrating the change across the salt boundary.

Over the last 15 years numerous modifications in detail have been introduced. Not all are improvements. In 1938 Philpot introduced a cylindrical lens into the optical system for Schlierin scanning which made it possible to watch the progress of separation of the components during an electrophoretic analysis. Longsworth (1942) described the single centre section which enables detailed analysis of mixtures to be made over the whole length of each limb.

Svensson (1946) described improved compensators by which the rate of forward migration of a protein could be controlled, and Hoch (1950) gave mathematical foundations to the considerations of "the steady state" by which the "purity" of a sample of a protein might be determined. Numerous modifications to electrode vessels and to the method of their junction with the U tube have been described (Svensson, 1946; Alberty, 1948). The U tube itself has been constructed successfully out of perspex instead of glass and the whole apparatus condensed in form (Longsworth and Alberty, demonstrated by Crook, El-Marsafy, Shooter, and Ward, 1952). The use of perspex may well materially reduce the cost of the apparatus, but the physical characteristics of perspex make it necessary either to reduce the temperature of the thermostatic bath or to lower the current densities at which analyses are carried out.

Finally a number of workers have described compact forms of apparatus using smaller amounts of material, having simplified thermostatic control, and modified cells (Moore and White, 1948; Stern and Reiner, 1946; Antweiler, 1947, 1949; Alexander and Saggers, 1948; Svensson, 1950) for routine analytical use. We believe that the majority of these micro modifications lose something of the original quality of the apparatus as ultimately developed by Tiselius (1937) and by Longsworth (1942).

Electrophoresis on Paper

The classical technique, as carried out in the apparatus designed by Tiselius or in the many modifications based on the original apparatus, is unlikely, by reason of its complexity, to be used widely in routine clinical laboratories in this country.

For this reason the relatively simple technique of paper electrophoresis requires special consideration. In comparing the two techniques it is always important to remember that, whereas in the classical technique one is measuring a series of zones of concentration superimposed successively one on the other, in the paper and allied techniques there is relatively complete separation of the individual components.

The Apparatus.—Numerous papers have been written on the design and construction of paper electrophoresis apparatus. The complexity of these designs varies enormously, ranging from the use of glass tumblers to elaborate constructions of "perspex." Nevertheless three distinct classes can be recognized: (a) those in which the paper is supported in the centre to form an inverted V; (b) those in which the paper or other medium is horizontal; and (c) those in which the paper or medium is hung or packed vertically.

Each of these classes has certain characteristics.

The Inverted V Type (Durrum, 1950; Flynn and de Mayo, 1951; Latner, 1952).—This type (Fig. 3) has been used widely in hospitals in Britain to separate serum and plasma proteins. One of its advantages over other types is that a technique can be acquired quickly which gives

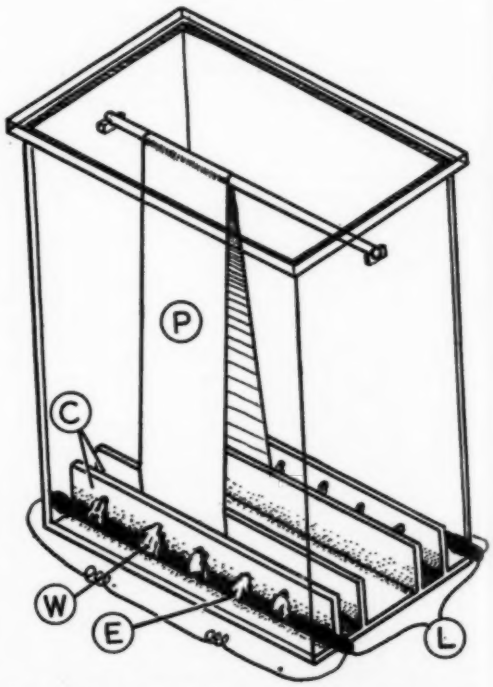

FIG. 3.—Diagram of the inverted "V" type of apparatus after Flynn and de Mayo. C=buffer chambers, E=electrodes, L=leads to electrodes, W="wicks" between buffer chambers, P=paper.

results sufficiently good for rough clinical analyses. It is unsuitable for the study of fundamental problems concerning electrophoresis, as conditions are too complex to control. In none of the apparatus of this type examined by the authors does the enclosed atmosphere reach a uniform temperature or vapour saturation. This is due partly to the limited surface of evaporation available to saturation of the relatively large air space, and partly to the temperature differences from the heating effect of the electric current. As a result, buffer continuously ascends the paper strip during a run, and is concentrated by the distillation of water vapour from the strip. Moreover, the arrangement of the strip results in the volume of liquid absorbed by unit quantity of paper being proportional to the height of that unit above the buffer level. The conditions therefore under which electrophoresis takes place are as follows: in addition to the electro-osmotic flow of buffer, there is also a "distillation" flow from the ends of the strip to the centre. At the same time there is an increase in the ionic strength of the buffer from the ends of the strip to the centre, a decrease in the volume of buffer held by the strip. It should be noted that such changes of ionic strength generally result in an alteration of the buffer *p*H. When buffers containing volatile constituents such as acetic acid are used, the conditions are complicated by the preferential loss of these constituents through increased evaporation as the centre is approached. This leads to a marked change in the *p*H of the buffer. Under these conditions it is impossible to attain a constant potential gradient along the strip, and it is not surprising that Durrum (1950) reported that a uniform rate of movement of albumin was not attained in his apparatus, a result we have been able to confirm. In another paper Durrum (1951c) reports that in prolonged electrophoresis amino-acids take up equilibrium positions which are unaffected by their original positions on the papers, and he explains this by consideration of the opposing effects on ionic migration of the buffer flow along the paper, and of the gradients in ionic strength of the buffer. The theoretical aspect of this system has been studied in detail by Macheboeuf (1953).

This type of apparatus has been adapted to two-dimensional separation, and by this means Durrum (1951c) has separated 11 out of a mixture of 19 amino acids, using acid and alkaline solvents. He also found that it was possible to obtain reproducible patterns from serum proteins by two-dimensional electrophoresis, but changes due to denaturation were liable to complicate the result.

The Horizontal Type.—This type (Fig. 4) has been developed from the original design of Cremer and Tiselius (1950). Several different versions have been described. The paper may be sealed from the air by being sandwiched between two glass plates so that evaporation from the paper strip is completely prevented (Cremer and Tiselius, 1950; Kunkel and Tiselius, 1951). In an effort to control the temperature of the strip more accurately Cremer and Tiselius immersed their sandwich in chlorobenzene. Recently Consden and Stanier described a model in which the buffer-soaked filter paper was immersed directly into chlorobenzene without glass plates (Consden and Stanier, 1952b). Other means of dissipating heat when running at high potentials have been described (Gross, 1953; Michl, 1952; Foster, 1952). In Weber's apparatus the paper rests on a glass plate, but the upper surface is exposed to the atmosphere of the box (Weber, 1951). In several models the paper hangs freely in the horizontal position (Wieland, 1948; Grassmann and Hannig, 1952; McDonald, Urbin, and Williamson, 1951a; Franglen, 1953). In all these models the horizontal position of the paper strip eliminates any effect that variation of height may have on the constancy of the buffer uptake. Even in those models in which the paper is not enclosed between glass plates or plunged in organic solvents, the saturation of the relatively small atmosphere is readily achieved. In this laboratory we have developed an apparatus in which the atmosphere about the freely hanging horizontal paper is maintained in a constant condition by the presence of buffer-damped filter paper supported just above and below the working paper (Franglen and Martin, 1954b). Rates of ionic migration are uniform both in the enclosed and the open type of horizontal apparatus, and this has led to attempts to formulate a mathematical theory of paper electrophoresis (Kunkel and Tiselius, 1951; McDonald *et al.*, 1951b). Durrum has pointed out, however, that "when paper is supported on one or both sides, even when the supporting surface is siliconed, a thin film of electrolyte lies at the interface between the paper and the supporting medium in which convection may not be effectively prevented and in which film the mobility may be different from that in the body of the paper" (Durrum, 1951b). This may be a cause of the trailing of protein components which is frequently encountered in techniques in which glass plates are used to confine the paper. Horizontal

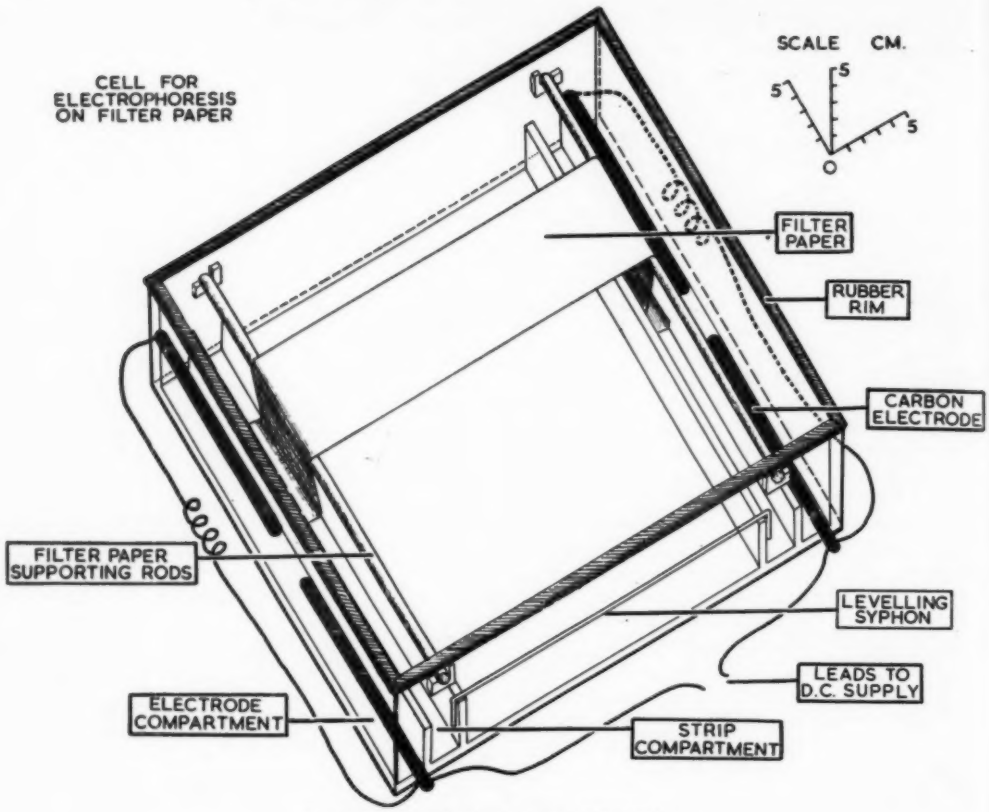

FIG. 4.—Diagram of a simple pattern of "flat" box.

apparatus has been adapted for use with other media such as starch (Kunkel and Slater, 1952a and b) or agar (Gordon, Keil, and Sebesta, 1949).

The Vertical Type.—Although a few models of this type for analytical work have been described, the majority have been designed for the continuous separation of components by zone electrophoresis (Haugaard and Kroner, 1948; Svensson and Brattsten, 1950; Brattsten and Nilsson, 1950; Grassmann and Hannig, 1950; Durrum, 1951a).

Preparative Methods Employing Electrophoresis.—The electrophoretic principle seems to present a means of separating proteins without seriously altering their structure.

A number of techniques embodying the principle have been developed. Many consist of modifications of the classical Tiselius apparatus so that zones containing specific migrating components may be removed without seriously disturbing the equilibrium of the remaining migrating boundaries (Maclagan and Bunn, 1947; Svensson, 1946; Kekwick, Lyttleton, Brewer, and Dreblow, 1951; Crook *et al.*, 1952).

These methods, although of great value to research workers, have little practical use in routine clinical laboratories. A certain number of methods have been developed in which both electrophoresis and convection have been used together to give a continuous technique for separation, and it is conceivable that the principles might be developed and applied on a commercial scale (Gutfreund, 1943; Gralén and Svedberg, 1941; Nielsen and Kirkwood, 1946).

Finally, Durrum applied a principle (Durrum, 1951a) originally developed by Svensson and Brattsten to electrophoresis (Svensson and Brattsten, 1950) on filter paper (Fig. 5). In his and

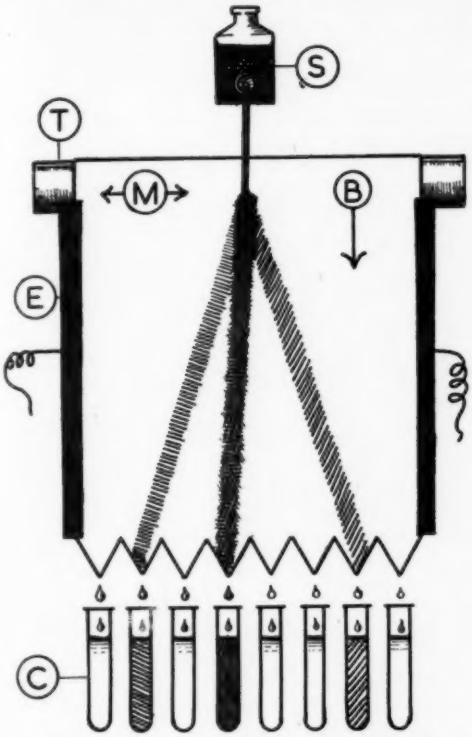

FIG. 5.—Diagram of the preparative method initiated by Durrum using electrophoresis on a vertical sheet of filter paper. B = direction of buffer flow, C = collecting tubes, E = electrodes, M = direction of applied current, S = reservoir containing solution of proteins for analysis, T = trough containing buffer.

Svensson's method a stream of buffer electrolyte is fed by gravity across a filter paper and at right angles to an electrical current passed through the filter paper.

If a solution of a mixture of proteins is allowed to flow gently from a point on the top edge of the paper it will be washed slowly down with the stream of buffer, moving under the influence of gravity. As the mixture passes down across the paper it will be subjected to the lateral impact of the electrical current moving at right angles to the direction of flow. This current will tend to pull apart the components of the mixture which will have separated, as they would in a simple electrophoretic run, when they reach the lower margin of the filter paper. The lower margin of the paper is serrated and a row of tubes conveniently placed collects the protein-containing buffer solutions as they drip off the paper in a series of fractions.

The original mixture is fed in from the reservoir at the top of the apparatus. The tubes collect the fractionated proteins at the bottom.

While the method may not have very wide application in clinical laboratories it is not very complicated or expensive to construct if at any time there is need for a preparative method.

A preparative model based on a slightly different principle has recently been described by Flodin and Porath (1954).

Necessary Conditions for Separation of Mixtures by Paper Electrophoresis.—Certain conditions for separating the mixtures are necessary and will be discussed.

Control of the Electrical Field : Power Input.—If consistent results are to be obtained a constant supply of D.C. with some simple form of voltage control is needed. A 120-volt high-tension dry battery similar to those used in old wireless sets is perfectly satisfactory. In normal daily use they should have a life of about four months and should then be renewed. A simple milliammeter should be included in series in the circuit. With the voltage adjusted to 120 to 150 volts a current density of 0.15 to 0.20 mA/cm. cross-section of paper should be obtained. Under these conditions in the flat pattern of box using reversible electrodes a satisfactory separation of serum proteins will be obtained on Whatman No. 1 filter paper using 0.05 molar barbiturate buffer at *p*H 8.6 in 12 to 16 hours.

If a more rapid separation is required, this can be managed by raising the voltage, but at higher voltages there is always a risk of distorting the pattern of the separating serum proteins unless special precautions are taken to prevent heating.

Some laboratories may have a D.C. supply and for those interested the circuit of a simple form of mains transformer incorporating a stabilizer is illustrated here (Fig. 6). This circuit was designed and used in this laboratory by R. G. O. Kekwick and has proved satisfactory.

Electrodes.—"According to the experience of the author reversible electrodes are quite indispensable" (Tiselius's thesis, 1930, p. 14). We agree that if satisfactory voltage control is to be achieved some simple form of non-polarizable electrode must be used in all forms of electrophoretic analysis.

We have found the silver/silver-chloride/potassium-chloride system, modelled in miniature from that originally designed by Tiselius for the classical apparatus, inexpensive, simple to construct, and reliable in use. Platinum electrodes

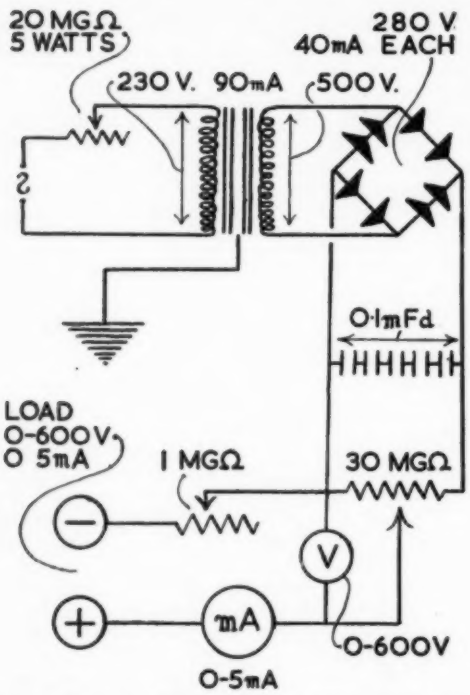

FIG. 6.—Diagram of mains transformer from A.C. to D.C. incorporating a stabilizer.

are equally satisfactory but are relatively expensive. Carbon electrodes are not reversible, polarize rapidly with a resulting tendency to *p*H shifts in surrounding buffer, and have a tendency to crumble. They are not satisfactory if any attempt is being made to achieve a steady field strength.

Gardell, Gordon, and Aqvist (1950) have used a platinum anode and a brass cathode.

Preparation of the Material for Electrophoresis.—The protein solution to be analysed should be dialysed against the buffer to be used before electrophoresis. This can be carried out using small cellophane bags, but does inevitably involve using more material than is normally available. In practice the serum to be analysed can be diluted with an equal volume of the buffer provided the final total protein concentration does not fall below 3 g. per 100 ml. In many instances satisfactory analysis may be obtained from direct application of the undiluted serum, but it is essential to make sure that there is no undissolved or precipitated material in the solution applied to the filter paper. Fibrinogen is particularly liable to precipitate and it is noticeable that for this reason plasma is frequently less satisfactory to run than serum.

Formation of the Starting Boundary.—Proteins are especially liable to denaturation if dried in the presence of oxygen or if spread over a large surface area.

The practice of applying the protein solution to the dry filter paper before the paper is saturated with buffer is likely therefore to lead to erroneous results. In like manner the use of paint brushes for the application of the serum should be avoided, as the contact of the protein solution with the paint brush, presumably due to the large surface area of the bristles, leads to denaturation.

It is our practice to soak a narrow ribbon of filter paper, already damped with buffer of the same quality as that in which the serum is to be analysed, in the serum and, having quickly and lightly blotted off the excess serum with buffer-damped filter paper, to place the ribbon immediately on the buffer-saturated filter paper which is to be used for analysis in position in the box. After contact for nine minutes the ribbon is removed with forceps, the box sealed, and the current started.

For quantitative application the solution is applied directly to the analysis paper using a micropipette or an "agla" micrometer syringe.

The Buffer.—To produce consistent analyses the buffers used should be adequate both in bulk and buffering capacity; the volume, to limit *p*H change from the migration of ions other than those being investigated; the capacity, to swamp the inherent buffering effects of the ions being investigated. Proteins themselves are powerful buffers and must be prevented from influencing the *p*H of the system.

The Buffer Volume.—In the classical apparatus in which the total volume of analysed material is about 10 ml. each electrode vessel holds 1,000 ml. In the classical preparative apparatus the electrode vessel volume is swollen to 5,000 ml. For filter-paper electrophoresis Kunkel and Tiselius calculated theoretically that to prevent *p*H change due to H_{ion} migration over 24 hours a minimal capacity of 313 ml. in each buffer chamber using barbitone is needed. Their apparatus had buffer chambers of 800 ml. capacity. While it is essential to have an adequate buffer volume, 800 ml. is probably a handsome margin of safety; indeed, in the apparatus we use, in which the electrode chamber is independent and linked by agar bridges to the buffer chamber, the buffer chambers themselves, into which the strips dip, have a volume of 300 ml.

each. *p*H measurements show no *p*H shift over 24 hours using silver/silver chloride electrodes, though if carbon is substituted the *p*H shift may be as much as 0.5 of a *p*H.

In certain apparatus described by Hall (1948) and Franglen (1953) the potential buffer capacity is increased by arranging the continuous flow of buffer through the buffer chambers.

Buffer Strength.—Buffer strengths giving satisfactory resolutions of protein mixtures range between 0.04 M and 0.15 M depending on the type of buffer used.

Increasing the concentration beyond these limits tends to reduce the effective mobility of the proteins and so impede separation. It increases the risk of precipitation and the risk of denaturation. Increased concentration of the buffer sharpens boundaries formed by limiting diffusion.

Decreasing the buffer concentration decreases the ability of the buffer to control *p*H, increases diffusion with blurring of boundaries, and tends to cause precipitation of the slower components. The optimum lies at about 0.12 M–0.06 M for barbiturate buffers at *p*H 8.6 and about 0.15 M–0.1 M for phosphate buffers at *p*H 8.0.

Buffer Patterns.—The choice of buffer pattern depends on the *p*H range at which the mixture is to be investigated. For most clinical purposes *p*H 8.6 using barbiturate buffer or *p*H 8.0 using phosphate buffer are the most suitable.

The constitution of the buffer affects the qualitative and quantitative separation of mixtures. Satisfactory comparisons cannot therefore be made between separations carried out in buffers that differ in constitution or concentration.

A simplified table of buffers we have found useful is given below (Table I). It should be noted that borate buffers, because of their capacity to form complexes, often produce unusual patterns with the α and β globulins.

Maintenance of Buffer Equilibrium.—It is essential to be certain that the box in which the analyses are being carried out is completely sealed and is free from air leaks. Any air leaks produce local disturbances in equilibrium with consequent distortion and irregularity of the patterns produced.

To prevent leaks we have used plasticine, or rubber washers and a screw-capped lid, with equal success. Mercury seals are satisfactory but expensive.

Hydrostatic Equilibration.—Before the passage of current in the ideal state, the buffer pattern and concentration should be uniform in the path of the analysis. In the classical apparatus there is no difficulty in arranging a stable buffer pattern in the chambers through which the boundaries are to pass. Special considerations arise when selecting buffer systems in the presence of cellulose fibres. These will be dealt with in the section on matrices.

Temperature Control.—For current densities up to 1.0 milliamps to 4 cm. width of strip, no appreciable temperature changes have been observed in the simpler patterns of apparatus for filter paper electrophoresis. In the apparatus we have used thermocouples embedded in the filter paper indicated a temperature fluctuation of 0.2° C. at 19° over 16 hours using silver/silver chloride electrodes and 1.5° C. using carbon electrodes, the runs being conducted at 5 milliamps and 100 volts.

If higher current densities are required the apparatus described by Cremer and Tiselius (1950) and modified by Schneider and Wallenius, in which

TABLE I
USEFUL BUFFERS

General Data			Data for Specific Concentrations and *p*H Values Named		
Constituents	*p*H Range	Concentration Range for Electrophoresis	Buffer Formula	Concentration	*p*H at 20° C.
Sodium acetate Acetic acid	4·0– 5·5	0·05–0·15 M	700 ml. N/10 sodium acetate 300 ml. N/10 acetic acid	0·1 M	5·0
Potassium dihydrogen phosphate Disodium hydrogen phosphate	6·0– 8·2	0·1–0·15 M	947 ml. M/10 Na_2HPO_4 53 ml. M/10 KH_2PO_4	0·1 M	8·0
Diethyl barbituric acid Sodium diethyl barbiturate	7·5– 9·0	0·05–0·12 M	10·3 g. diethylbarbituric acid 1·84 g. sodium diethyl barbiturate in 1 litre of water	0·06 M	8·6
Boric acid Potassium chloride Sodium hydroxide	7·8–10·0	0·05–1·0 M	(A) 12·37 g. H_3BO_3, 14·91 g. KCl, both in 1 litre water (B) 428 ml. 4% caustic soda Add (A) to (B) and mix	0·05 M	9·0
Glycine Sodium chloride Sodium hydroxide	8·5–12·0	0·05–0·15 M	(A) Glycine 4·55 g., NaCl 3·53 g., in 600 ml. (B) 0·4% caustic soda, 400 ml. Add (A) to (B) and mix	0·1 M	10·0

the paper is clamped between glass plates and the plates immersed in chlorobenzene, has proved satisfactory (Schneider and Wallenius, 1951). The modification of this technique introduced by Kunkel and Tiselius (1951), in which the glass plates are sealed in silicone grease and not plunged in chlorobenzene, has been adapted ingeniously by these authors. The whole apparatus was placed in a cold room, the glass plates resting on a brass block. By this means adequate heat dispersion was obtained and voltages from 80 to 400 volts giving current ranges from 4 to 20 mA were used satisfactorily. These levels approach those commonly used in the classical apparatus.

The Filter Paper Matrix.—Only the best grades of paper should be used for analysis, preferably those "for chromatography." We have found Whatman Nos. 1 and 4, Whatman 3 MM., and Munktell 20 absolutely satisfactory for all general uses. Since the rate of migration, and therefore the degree of effective separation in a given time, is affected by the texture of the paper, comparisons of analyses made on different types of paper should be avoided.

Consden and Stanier (1952b) have compared 11 grades of Whatman paper using albumin as a reference substance. Their data is given in tabular form below.

MIGRATION OF HUMAN SERUM ALBUMIN AND ELECTRO-ENDOSMOSIS

(cm./hr. on various Whatman papers in borate buffers *p*H 8·6 at 10·3 V/cm. at 20° C.)

Whatman No.	Electro-endosmosis	Migration of Albumin
1	0·9	3·2
2	0·9	2·8
3	0·9	3·4
4	0·6	2·8
5	0·4	2·1
20	0·9	2·7
42	0·7	2·4
50	0·2	1·4*
52	0·7	2·1
54	0·8	2·4
542	0·5	1·6*

* Not measurable accurately: papers unsuitable for serum proteins.

The filter-paper matrix cannot be regarded as a neutral support medium. It carries a measurable negative charge. Many attempts have been made to neutralize this charge using gentian violet (Hauser and Lynn, 1940), "cetab" (Rutter, 1950), and albumin (Tiselius, 1953). Jermyn and Thomas (1953) have studied the endo-osmotic flow using spots of tri-nitro benzene and O. nitroaniline. Finally at extreme acid and alkaline *p*H ranges in the region of *p*H 3 and *p*H 11 the cellulose fibres themselves fragment and swell, making analysis in these regions difficult if not impossible.

Methods of Analysis.—One of the most important steps in electrophoretic analysis is the technique by which the boundaries, once established, are identified and by which the composition of the separating mixture is expressed in quantitative terms. Each method of estimation has its special difficulties, which not only define the limitations of the analysis incorporating the method, but limit the comparisons which may be made with analyses made by alternative methods.

As most of the mixtures analysed by paper electrophoresis are invisible on the supporting media, special procedures are necessary to determine the position of the separated components. Many such procedures have been described, of which a number have been adapted to give quantitative estimation of the components, in addition to their localization. The most usual method of locating proteins on paper "electrophoretograms" is by visualization with dyes, either of the acid wool or of the indicator type.

The procedure commonly adopted is to remove the paper strip after completion of the electrophoretic run, taking care in removing it not to allow it to brush against the apparatus or the bench. The paper is then suspended in an oven and dried at 105° C. for 30 minutes. The paper may then be dyed by a number of techniques. Four of these in common use in this laboratory are given in Table II.

Countless dyeing procedures, however, have been described in attempts to obtain the maximum contrast between the dyed protein and the background, and to obtain the maximum uptake of dye by the different protein fractions. These procedures have been extended to the analysis of protein separated on gels, since, by applying a piece of filter paper to the surface of the gel, a protein print is obtained which can be dyed by the usual methods (Consden, Gordon, and Martin, 1946).

Substances which can be located on the supporting medium by their fluorescence or absorbence in ultra-violet light may be identified directly (Paladini and Leloir, 1952). Attempts have been made to use such fluorescence for the quantitative estimation of separated components, and special apparatus has been designed for this purpose (Hashimoto and Mori, 1952; Semm and Fried, 1952).

Some components separated by paper electrophoresis can be located and estimated by their specific biological action. This approach has been particularly useful in the study of enzymes and hormones, activity being located either by incubation *in situ* with chromogenic substrates (Mills

TABLE II
COMMONLY USED DYEING TECHNIQUES

Dye Solutions	Time of Dyeing (min.)	Washing Solution	Drying	Adapted From
Bromophenol blue, 1 g. EtOH sat. with $HgCl_2$, 100 ml.	5–10	0·5% acetic acid. Four successive washes over 20 minutes until background is clear	Dry at lab. temp.	Kunkel and Tiselius, 1951 (Cremer and Tiselius, 1950)
Azocarmine B, 0·75 g. MeOH, 50 ml. Acetic acid glacial, 10 ml. Water up to 100 ml.	10	1st wash 90% methyl alcohol containing 10% acetic acid v/v. Final wash in 10% acetic acid in water	Dry at lab. temp.	Plückthun and Götting, 1951 (Turba and Enenkel, 1950)
Bromocresol green, 0·2 g. Acetic acid glacial, 2 ml. 95% EtOH up to 100 ml.	10	2·0% acetic acid in water. Four successive washes over 20 minutes until background is clear	Dry at lab. temp. and "blue up" over conc. ammonia bottle	Franglen, 1953
Naphthalene black 12B, 0·1 g. Acetic acid glacial, 10 ml. Methyl alcohol, 90% to 100 ml.	10	1st wash 90% methyl alcohol containing 10% acetic acid v/v until background is pale blue. 2nd wash pure methyl alcohol 5 minutes	Dry at lab. temp.	Flynn and de Mayo, 1951 (the Amidoschwartz 10 B of Grassmann *et al.*, 1951)

Ponceau red 2R. (Röttger, 1952) and light green C.I. 670 (Smith and Dangerfield, 1954) have also proved very satisfactory.

and Smith, 1951) or by standard methods of assay after elution from the supporting medium (Kunkel, Taylor, and du Vigneaud, 1953). It is, perhaps, not superfluous to point out that, if studies of this nature are to be of real value, it is essential to check that such activity is not altered by the conditions necessary for electrophoresis.

In several methods, a component of the mixture is marked with radioactive tracers or dyes before electrophoresis, so that it can be located on the supporting medium during or after separation. Radioactive iodine (Larson, Deiss, and Albright, 1952), iron (Wallenius, 1952a), phosphate (Maurer and Schild, 1951), and labelled amino-acids (Gros, Dubert, Sung, Coursaget, and Macheboeuf, 1952) have all been incorporated into proteins to aid in their identification after separation. N-tolyl alpha-naphthylamine 3 sulphonic acid combines with albumin to give a brilliantly fluorescent complex which we have used for following the migration of albumin on filter paper. Other workers have found the dyeing of serum albumin before electrophoresis with bromophenol blue (Kunkel and Tiselius, 1951) or bilirubin (Flynn and de Mayo, 1951) a useful means of judging the degree of separation of serum proteins in cells in which the speed of separation is liable to variation. It is a little surprising to note that the mobility of these albumin complexes is not significantly different from the untreated albumin. Radioactive iodinated albumin has been reported to have a higher mobility than non-iodinated albumin (Gabrieli, Goulian, Kinersly, and Collet, 1954). These observations needed to be checked with other protein-tracer complexes, and until such checks have been completed conclusions based on pre-marking techniques must be treated with reserve.

Comparisons of the dyed protein electrophoretograms with classical Schlierin diagrams suggested to earlier workers that determination of the protein-bound dye would form a means of estimating the relative proportions of the protein fractions (Cremer and Tiselius, 1950; Flynn and de Mayo, 1951). In many of the earlier methods the dyed filter paper electrophoretogram is cut into consecutive strips of equal width from which the dye is eluted by alkaline solutions or organic solvents and measured by absorptiometry (Cremer and Tiselius, 1950; Robinson and Fehr, 1952; Loeffler and Wunderly, 1953). For accurate dye determination it is essential that fading does not occur in the eluting solution (Franglen and Martin, 1954a). Although simple in use, these elution procedures are tedious and cannot be applied to every dye.

Methods of measuring the optical density of the protein-dye complex on the paper directly were developed to overcome these shortcomings, and vary in complexity from the use of visual matching techniques (Latner and Richardson, 1952) to the use of elaborate self-recording photoelectric "scanners" (Crook, Harris, and Warren, 1952; Einsenreich and Eder, 1951; Grassmann, Hannig, and Knedel, 1951; Griffiths, 1952; Latner, Braithwaite, and Nunn, 1952; Latner, Molyneux, and Rose, 1954; Van Os, 1952, 1953; Weicker, 1953). Many of the earlier "scanning" methods, such as that of Grassmann and his co-workers, have a disadvantage in that clarification of the paper with paraffin mixtures is necessary before measurements can be made, but recently this has been overcome by the photoelectric measurement of light reflected from the dry paper (Röttger, 1953). Many of the simpler "scanners" have an inherent weakness in that no compensation is made

for the decrease of photocell response with time, and in such cases, unless scanning is rapid, results cannot be truly reproducible. Michl has described a different method of optical evaluation based on the use of an absorption wedge with a cylindrical lens system (Michl, 1952) which, although simple, does not suffer from this defect.

Both types of dye analysis produce graphical plots which bear a superficial resemblance to Schlierin diagrams obtained on the classical apparatus (Fig. 7). Rigid comparison with

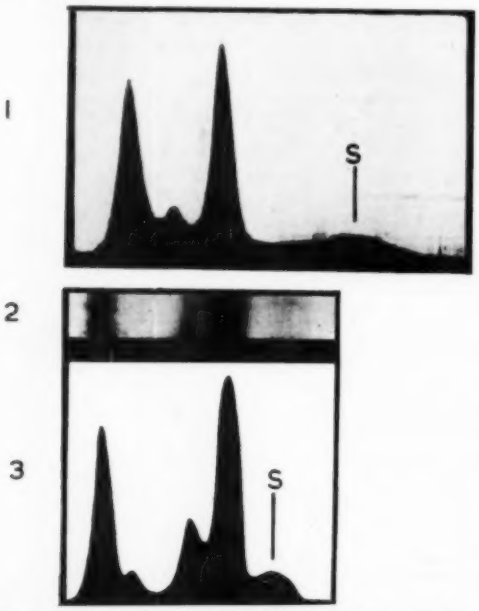

FIG. 7.—Analyses of the serum of a patient suffering from myelomatosis indicating the qualitative and quantitative differences that may occur between the classical technique and paper electrophoresis. 1 is the Schlierin pattern on the Tiselius apparatus in 0.1 μ barbiturate buffer at *p*H 8.6. 2 is the "electrophoretogram" in the flat box in 0.06 M barbiturate buffer at *p*H 8.6 stained with azocarmine B. 3 is the analysis of the electrophoretogram (2) by reflected light in an automatic scanner. S indicates the starting boundary in each analysis.

classical electrophoresis showed that differentiations achieved by the old method were not constantly mirrored in the new. The variations in mobilities obtained with purified fractions, whether using dextran or albumin markers, indicated that these could only form the crudest means of identification (Martin, 1952). Moreover, the distribution of dye between the separated protein fractions is not identical with the proportions of the fractions as determined by the refractive index method (Cremer and Tiselius, 1950). A great deal of effort has gone into devising correction factors or modifications which would enable analyses by the younger method to approximate to the older (Cremer and Tiselius, 1950; Köiw, Wallenius, and Grönwall, 1951, 1952; Kunkel and Tiselius, 1951; Levin and Oberholzer, 1953; Schneider, 1951), and many procedures of determining serum and other protein fractions by dye estimation have been described (Antonini and Piva, 1952; Esser, Heinzler, Kazmeier, and Scholtan, 1951; Goa, 1951; Grassmann *et al.*, 1951; Körver, 1950; Loomeijer, 1952; Michl, 1951, 1952; Noverraz, 1952; Plückthun and Götting, 1951; Vignato, 1952; Vishnyakov, Dobrovol'skii, Ermakov, and Tuckachinskii, 1952).

These procedures assume primarily that all the proteins examined will interact with the chosen dye, that the dye uptake bears a simple relationship to the protein concentration, and that the dye-binding capacities of the different fractions, although not necessarily identical, will bear a simple consistent relationship to each other at all concentrations at which each component is met. Unfortunately none of these assumptions has proved to be true; "correction factors" may vary by several hundred per cent, and the uptake of dye is not always a linear function of protein concentration (Figs. 8a and 8b) (Köiw *et al.*, 1952; Grönwall, 1952; Crook, Harris, Hassan, and Warren, 1954; Franglen and Martin, 1954b). Moreover, since dye samples often vary considerably in composition from maker to maker and from batch to batch (Tudor Jones in a paper read before the Fine Chemicals Group of the Society of the Chemical Industry on January 15, 1954) it is unlikely that correction factors will remain constant for any one dye even with rigorously controlled protein concentrations. It must be concluded therefore that the present dye methods are not generally suitable for the accurate estimation of protein fractions. At the same time, this does not prevent these methods continuing to be of clinical value in indicating gross changes in individual protein components.

In order to eliminate the uncertainties inherent in the analysis of proteins by dyeing procedures, other methods of analysis have been described. Nitrogen determinations on the separated protein fraction by Kjeldahls have been undertaken by some workers (Anderson, 1952; Levin and Oberholzer, 1952, 1953). Since the quantity of protein nitrogen involved is small, electrophoresis of the serum is usually carried out in non-nitrogenous buffers, such as phosphate or borate, in which fractionation tends to be poor. Although the accuracy claimed for these procedures is high, their tediousness does not recommend them for general use.

Kunkel and Tiselius (1951) have adapted the Heidelberger and MacPherson (1943) procedure for the determination of tyrosine to the estimation of proteins separated by paper electrophoresis. The use of Folin's reagent as a means of quantitative assessment of individual proteins has been criticized on the basis of the known variations in tyrosine content that occurs between different protein species (Martin, 1952). The phenomenon of ion retention has been used for the analysis of protein and other types of electrophoretograms (Wieland, 1948; Wieland and Fischer, 1948; Wieland and Wirth, 1950; Robinson and Fehr, 1952). In analysis by this method the edge of the electrophoretogram parallel to the direction of migration is dipped into a coloured solution

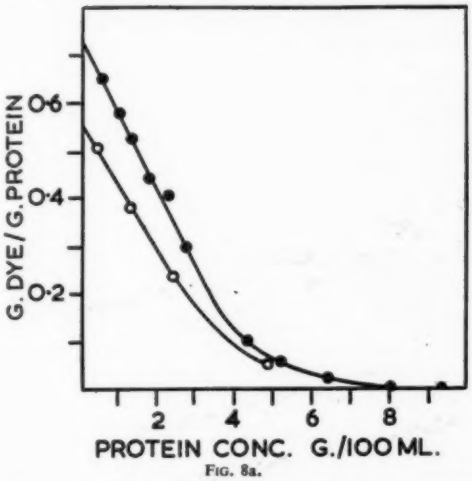

FIG. 8a.

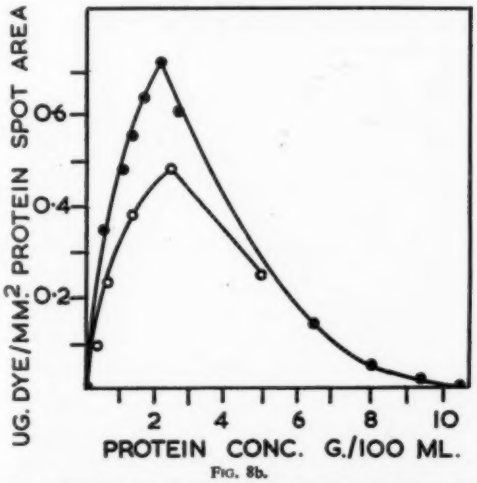

FIG. 8b.

FIG. 8a and 8b.—Curves of the uptake of bromo cresol-green by human proteins on paper. Fig. 8a illustrates that using elution methods the uptake of dye by unit weight of protein is not constant. Fig. 8b illustrates that the uptake of dye by unit area of protein (the basis of scanning techniques) only varies directly with protein concentration at low concentrations. -o-o-o- γ globulin. -●-●-●- albumin.

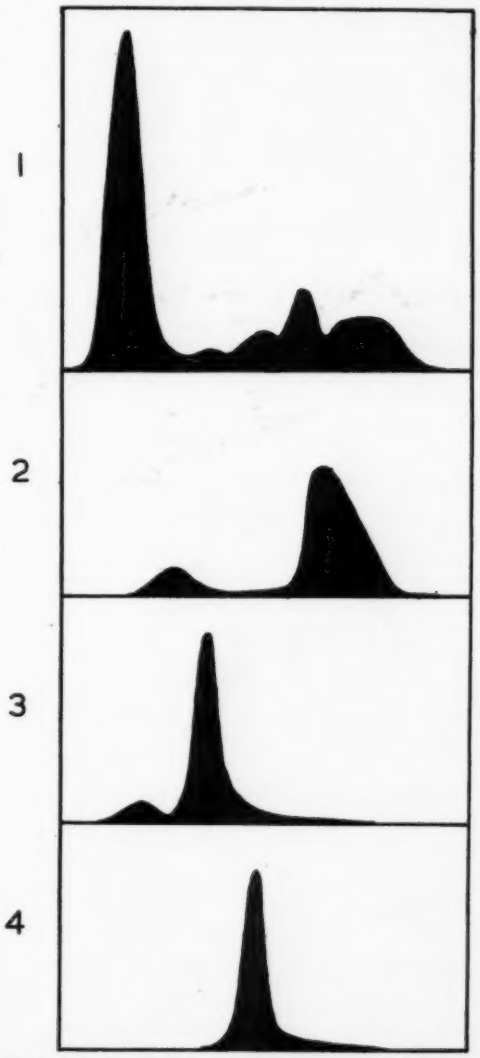

FIG. 9.—Serial analyses of a normal serum showing the position in the protein mosaic of some of the constituents. 1=the serum proteins, 2=the lipoproteins, 3=the distribution of radioactive iodine, 4=the distribution of radioactive iron.

such as that of copper acetate, erythrosine, or Solway purple. As the solution is soaked up by the paper, the coloured component combines with the protein, causing the formation of an irregular front. The areas of the irregularities are claimed to be simply related to the protein present, but Robinson and Fehr (1952) have shown that such relationships cannot be found with all proteins, and, in our experience, results obtained by retention analysis are very approximate, and tend to be less satisfactory than those obtained by a visual inspection of the dyed electrophoretogram.

Visualization of lipid fractions (Fig. 9) have been reported by several workers (Nikkilä, 1952 ; Swahn, 1952 ; Wunderly and Pezold, 1952). Both Sudan III (Fasoli, 1952, 1953 ; Kunkel and Slater, 1952a) and oil red O (Durrum, Paul, and Smith, 1952) have been used, and we have found Sudan black to be very satisfactory. It must be pointed out, however, that fat stains are notoriously variable in reaction, and we have had several batches of dyes which have proved to be quite useless for staining lipid on paper. In connexion with this class of substances, it should be mentioned that Kunkel and Slater found that the fractionation of serum lipid was improved by the use of buffers of low ionic strength or by the use of starch in place of paper as a supporting medium.

Protein-bound carbohydrates have been visualized by a procedure involving the use of Schiff's reagent (Köiw and Grönwall, 1952).

Other substances have been analysed by techniques originally developed for paper chromatography. These include amino-acids and peptides (Biserte, 1950 ; Consden and Gordon, 1950 ; Durrum, 1950, 1951c ; Kickhöfen and Westphal, 1952 ; McDonald *et al.*, 1951a and b ; Michl, 1951 ; Reindel and Hoppe, 1953 ; Suzuki and Maekawa, 1951) and carbohydrates (Consden and Stanier, 1952b ; Foster, 1953 ; Foster and Stacey, 1953 ; Gross, 1953).

Applications.—For brevity we have used tables (Tables III, IV, and V) to give some indication of the way in which the technique has been applied. The tables speak for themselves, and it is only necessary to comment on pitfalls and limitations in routine use.

Qualitative and semi-qualitative analysis of protein-containing fluids can be made effectively by the method if the protein concentration is above 2%, provided care is taken to remove *all* insoluble material and to remove excess of soluble salts either by dialysis or by desalting (for details of the technique see Consden *et al.*, 1947). Any procedures carried out on protein solutions at room temperatures are liable to cause some denaturation. Extreme caution is therefore needed in interpreting subsequent analyses unless the most scrupulous precautions can be and are taken. We do not believe that desalting is a technique suitable for routine laboratories, nor that the number of times it is needed would justify the expense of the apparatus. For most protein-containing fluids dialysis is sufficient.

For protein solutions below 2 g. per 100 ml. some form of concentration is usually desirable. Three relatively simple methods are available, ultrafiltration, dialysis against strong solutions of dextran or some other suitable polymer, or acetone precipitation. The last method is undesirable because of the very great risk of denaturation. The choice between the first two depends on convenience and the amount of available material.

All methods involve a considerable expenditure of time and skilled labour if the results obtained are to be consistent and reliable. It is doubtful, in the present state of knowledge, whether the useful information they give is so far in advance of that obtained by older and simpler techniques as to justify their regular use in the majority of routine clinical pathological laboratories.

We have observed discrepancies repeatedly between analysis by the classical technique and by paper electrophoresis. We believe that the classification of any disease on the basis of analyses that show such inconsistency is a fruitless procedure until more is known of the factors which produce these inconsistencies.

Conclusions

Because of the more meticulous approach to the classical technique initiated by the pioneer work of Tiselius, chaos has not and is not likely to occur using that method. Its great value is likely to remain as a tool of research and reference. Because of its cost, the amount of material required for analysis, and the time taken for each analysis, it will probably remain an unsuitable technique for the majority of routine clinical laboratories in the foreseeable future. For biological problems where limited material is available, multiple analyses are required, and speed is a consideration, the method of electrophoresis using paper seems admirably suited.

It is unlikely in our present state of knowledge that all the theoretical requirements can be met by any one design, but that should not be made a bar to progress, nor should it be an excuse for neglecting those requirements which can be met with reasonable simplicity.

TABLE III

ANALYSES OF CLINICAL SUBSTANCES

Substance	Supporting Medium	Method of Analysis	References
Normal and pathological serum proteins	Paper	Dyes	Flynn and de Mayo (1951); Köiw *et al.* (1951); Macheboeuf *et al.* (1951); Gras (1952); Köiw and Grönwall (1952); Loomeijer (1952); Noverraz (1952); Sternberg (1953); Wunderly (1953)
Serum proteins in multiple myelomatosis	,,	Dyeing with bromophenol blue	Griffiths and Brews (1953)—a study of 20 cases
Serum proteins in liver and bile disease	,,	Bromophenol blue	Brante (1952)
Cerebrospinal fluid proteins	,,	Dyes	Schneider and Wallenius (1951) concentration by dialysis against dextran solutions; Bucher *et al.* (1952a and b) concentration by acetone precipitation of protein; Caspani (1952); Esser and Heinzler (1952) concentration by ultrafiltration; Esser *et al.* (1952); Wallenius (1952b) normal and pathological c.s.f.; Mies (1953); Peters (1953); Plückthun and Matthes (1953)
Urinary proteins ..	,,	,,	Macheboeuf *et al.* (1951); Slater and Kunkel (1953); Squire (1953) distribution and clearance of protein in nephritis
Lipoproteins	Paper and starch	Lipid dyes	Durrum *et al.* (1952); Fasoli (1952, 1953); Kunkel and Slater (1952a) use of starch as a supporting medium; Nikkilä (1952); Swahn (1952); Wunderly and Pezold (1952)
Serum protein-bound carbohydrates	Paper	Schiff's reagent	Köiw and Grönwall (1952)

TABLE IVa

MISCELLANEOUS ANALYSIS OF CLINICAL INTEREST (a)

Substance	Supporting Medium	Method of Analysis	References
Haemoglobins	Paper	Dyes	Larson and Ranney (1953) normal and sickle haemoglobin; Ranney *et al.* (1953) haemoglobin C; Smith and Conley (1953); Spaet (1953)
Phosphorus metabolism of erythrocytes	,,	P^{32}	Schild and Maurer (1951, 1952)
Proteins of crystalline lens	,,	Dyes	Choisy *et al.* (1952)
Antibodies	,,	Use of valine C^{13}, antigenic activity	Gros *et al.* (1952) speed of renewal of antibodies; Janković and Krijnen (1953) localization of activity of anti-D sera
Renewal of serum proteins	,,	Use of methionine S^{35}	Niklas and Maurer (1952)
" Organic phosphorus " in serum	,,	Use of radioactive phosphorus P^{32}	Maurer and Schild (1951); Maurer (1952) serum " organic phosphorus " is associated mainly with albumin
Serum iron	,,	Use of radioactive iron	Wallenius (1952a) serum iron is associated with beta globulin; Horst and Schäfer (1953)
Nucleic acid hydrolysates	Paper, agar gel, and copper silk fibre	Fluorescence under ultraviolet light, reaction with molybdenum blue or fuchsia, labelling with radioactive phosphorus	Kanngiesser (1951); Smellie and Davidson (1951); Wieland and Bauer (1951); Deimel and Maurer (1952); Dimroth *et al.* (1952); Edström (1952, 1953) analysis of ribonucleic acid hydrolysate from individual nerve cells

TABLE IVb

MISCELLANEOUS ANALYSIS OF CLINICAL INTEREST (b)

Substance	Supporting Medium	Method of Analysis	References
Phosphate derivatives of adenosine	Paper	Ultra-violet	Turba and Enenkel (1951) use of acetate buffer; Jaenicke and Vollbrechtshausen (1952) separation as borate complexes
Carbohydrates	Paper and silk	Usual chromatographic techniques	Consden and Stanier (1952a) separation as borate complexes; Jaenicke and Vollbrechshausen (1952); Michl (1952); Foster (1953); Foster and Stacey (1953); Gross (1953); Geldmacher-Mallinchrodt and Weinland (1953) glycogen and galactogen
Acid mucopolysaccharides	Paper and supercel	Toluidine blue	Rienits (1953); Gardell *et al.* (1950)
Animal serum proteins ..	Paper	Dyes	Ganzin *et al.* (1952); Scheiffarth and Berg (1952)
Avian serum proteins ..	,,	,,	Common *et al.* (1953)
Amino-acids and peptides	Paper, silicia, gel, agar	Usual chromate graphic techniques	Consden *et al.* (1946); Biserte (1950); Consden and Gordon (1950); Durrum (1950, 1951c); McDonald *et al.* (1951a and b); Michl (1951); Suzuki and Maekawa (1951); Kickhöfen and Westphal (1952); Reindel and Hoppe (1953); Strange and Harkness (1953)
Cytochrome C	Paper		Paléus (1952)
Higher fatty acids ..	String	Formolized bromocresol purple	Barnett *et al.* (1953)

TABLE Va

BIOLOGICALLY ACTIVE SUBSTANCES OF CLINICAL INTEREST (1)

Substance	Supporting Medium	Method of Analysis	References
Enzymes in cell extracts	Paper	Biological activity	Wallenfels and Pechmann (1951) analysis of amylases, proteinases, lipases and phosphatases; Gillespie *et al.* (1952) analysis of esterases and amylases
Digestive enzymes ..	"	" "	Merten *et al.* (1952) analysis of pepsin; Nikkilä *et al.* (1952) analysis of trypsin; Heinrich (1953) analysis of pepsin
Serum cholinesterase ..	"	Biological activity and chromogenic substrates	Kekwick (1953) cholinesterase activity associated with at least two of the serum protein fractions
Beta-glucuronidase ..	Paper and agar	Chromogenic substrates	Mills and Smith (1951)
Hyaluronidase	Paper	Staining and activity	Caputo (1954) separation of hyaluronidase from inactive impurities
Vitamin B_{12}	"	Biological assay	Latner *et al.* (1952) vitamin B_{12} in serum; Schweitzer and Wittern (1952) vitamin B_{12} in liver extracts
Growth factors	"	" "	Ericson *et al.* (1952) factors for various lactobacilli
Pituitary extracts ..	Paper, starch	Dyes, activity	Michl *et al.* (1951); Li (1952) A.C.T.H. preparations consist of several different fractions; McDonald and Marbach (1952); Kunkel *et al.* (1953) oxytocin; Loomeijer and Witter (1953); Thing *et al.* (1953); Li *et al.* (1954)

TABLE Vb

BIOLOGICALLY ACTIVE SUBSTANCES OF CLINICAL INTEREST (2)

Substance	Supporting Medium	Method of Analysis	References
Circulating thyroid hormone	Paper and starch	Radioactive iodine	Deiss *et al.* (1952) i.v. iodine is associated with slow-moving albumin and alpha one globulin; Larson *et al.* (1952, 1954) iodine associated with alpha one globulin is in the form of thyroxin; Gordon *et al.* (1952); Maurer and Reichenbach (1952); Robbins and Rall (1952); Horst and Rösler (1953)
Antibiotics	Paper	Reactivity	Hosoya *et al.* (1951); Kutzim (1952a and b) analysis of sulphonamides; Foster and Ashton (1953) streptomycins; King and Doery (1953)
Bacitracin	"	"	Porath (1952) purification
Madronin	Agar gel	"	Hammarlund and Rising (1953) separation from partially purified antibacterial mixture
Plant viruses	Paper	"	Gray (1952)
Diphtheria toxin and antitoxin	Agar gel and paper		Field and Teague (1907); Poulik (1952)
Snake toxin	Paper	Visual inspection and dyes	von Klobusitzky and König (1939) separation of yellow pigment from snake venom; Grassmann (1951) fractionation of five protein-like components in snake toxin
Animal toxins	"	Activity	Wieland, Wirth, and Fischer (1949); Neumann and Habermann (1952)

It is within the capacity of any laboratory to use and maintain simple reversible electrodes. By proper planning of work, time can be allotted in any laboratory for buffer equilibriums to be established before protein boundaries are made and the run started. With a simple apparatus reasonable and reproducible qualitative comparison can be obtained between successive analyses (Fig. 10). But equally, neglect of fundamental principles will result in the production of unusual results arising from the technique and not the sample examined.

So much for the procedure of separation. The analysis of the separated components is more difficult, but if too much is not made of the quantitative aspect of the results, useful diagnostic information can regularly be obtained by the simple procedure of scanning a normal serum with each batch of unknowns and confining reports to semi-quantitative comments.

The section on applications will have indicated the potential range of usefulness in fields of research. It may be assumed that research workers will apply and describe their own rigorous checks and controls for each problem as it arises.

It is difficult in such a brief review to give weight to all aspects of the subject. It is hoped that this survey with its appended bibliography will be some help to those interested in this fascinating field and will warn others of the pitfalls.

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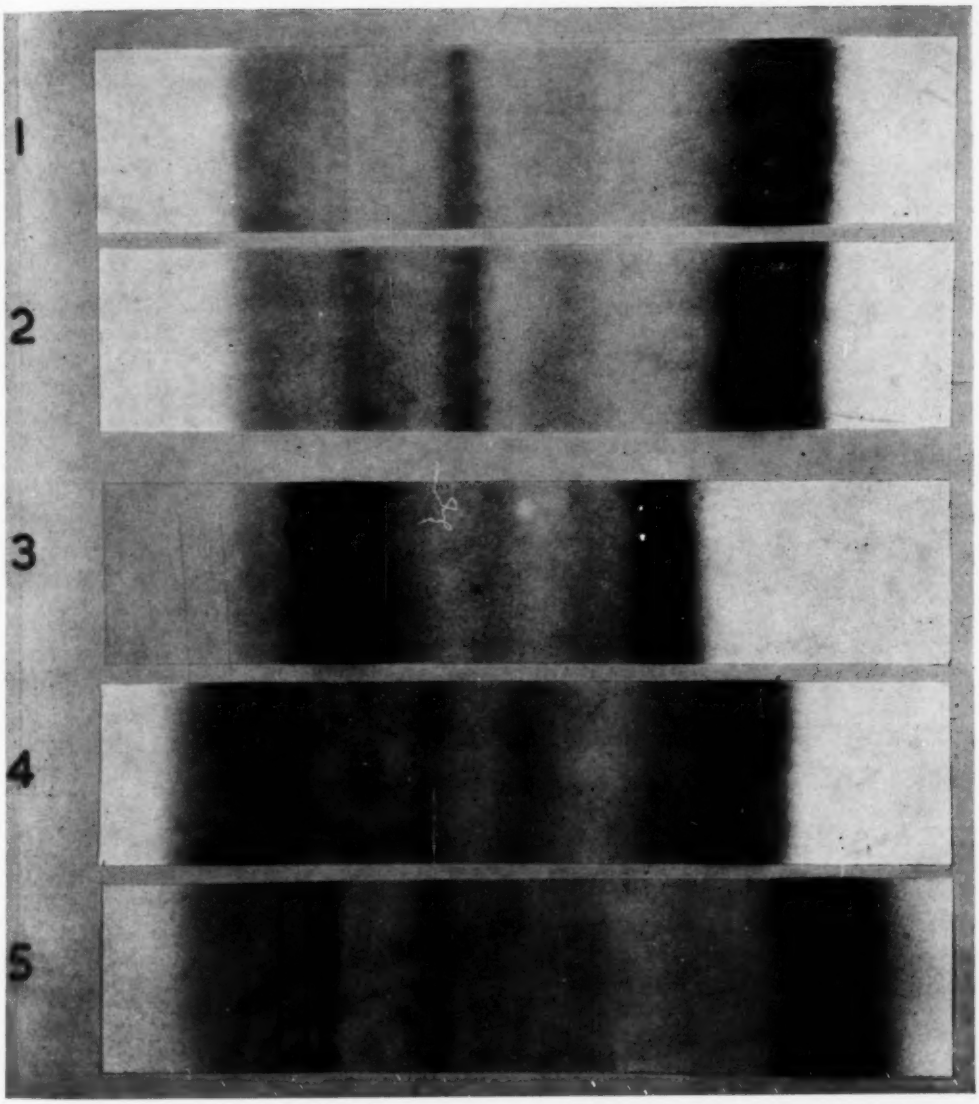

FIG. 10.—Analysis of normal and abnormal human circulating proteins using the filter paper technique on the flat box. 1 = normal serum, 2 = normal plasma, 3 = γ type multiple myeloma serum, 4 = chronic lymphatic leukaemia serum, 5 = hypercholesterolaemia serum.

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THE ESTIMATION OF γ-GLOBULIN

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WITH STATISTICAL ASSISTANCE FROM

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Filter-paper electrophoresis appears to be a technique suitable for the routine laboratory; the apparatus is simple and cheap, and although the electric current must be applied for 12 to 24 hours it is easy to arrange for this to occupy the night. During the last two years we have studied this technique, and conclude that the advantages are to some degree illusory. By this we do not mean that the information gained is useless, but that much of the useful information can be obtained by less laborious methods.

Martin and Franglen on page 87 of this issue write a critical review of the subject, and no detailed bibliography will be given. We must emphasize that in classical electrophoresis protein concentrations are estimated from differences in refractive index: this is a non-specific property, and is affected by serum-lipoids which migrate chiefly with the α_2- and β-globulins, thus rendering estimates of the protein components fallacious. On filter-paper, the choice of a suitable dye should enable one to estimate true protein values (Loeffler and Wunderly, 1953).

It is easy, with any one of a series of dyes, to produce an electrophoresis strip which permits visual estimation of the proportions of the different protein components. It is much more difficult to obtain an objective measure of these protein components: the most reliable, but most laborious methods, are those in which the dye is eluted from segments of strip (Loeffler and Wunderly, 1953). Scanning methods, in which the light transmitted by the strip is measured, are less laborious but less reliable, for the very complex optical problem, in which absorption by dyestuff is superimposed on absorption and scatter by cellulose fibre immersed in air ($\mu = 1.0$) or organic solvent ($\mu = 1.4 - 1.7$), has been studied only by Crook, Harris, Hassan, and Warren (1954).

We concluded that there was a need for the examination of a very simple technique, and this paper describes such a technique and some of the results obtained. The most important result is that we find a very close correlation ($= +0.9143$ based on 45 pairs of observations) between Kunkel's (1947) zinc sulphate turbidity test and our electrophoretic estimates of γ-globulin. This agrees with Kunkel's own results when using classical electrophoresis. Since it is well known that changes in γ-globulin occur in a great variety of diseases, including liver disease, rheumatoid arthritis and other collagen diseases, some reticuloses, kidney disease, and myelomatosis, we consider that Kunkel's test is the most important of the non-specific "turbidity" tests, since it gives so close an estimate of γ-globulin, and we think that for most routine purposes it should do away with any need for electrophoresis.

Procedure

Electrophoretic Separation.—A strip of Whatman No. 1 filter paper, 5 cm. wide and 50 cm. long, is fixed in a perspex holder so arranged that the electric current is led in on two lines 45 cm. apart. A middle section, 28 cm. long, is held horizontal and approximately 7 cm. above the buffer solution in the electrode vessels. The buffer solution is sodium barbitone, 0.05M, and barbitone, 0.01M, saturated with chloroform. When the paper has been placed in electric contact, soaked with buffer and allowed to come into gravitational equilibrium, serum (0.02 ml.) is applied in a streak about 45 mm. long, approximately 10 cm. from the negative end of the horizontal paper. The electric current is switched on (175 to 200 volts, 0.6 mA per paper) and left on for 20 to 22 hours.

Fixing and Staining.—The wet strip is cut off the holder and dropped into the combined fixing and staining solution, containing 0.133% light green S.F. (Lissamine Green S.F. 130 from I.C.I. Dyestuffs, Ltd., colour index 670) in 3% salicylsulphonic acid. It is left for between 30 and 120 minutes, and then washed for five minutes or more in each of three changes of

½–2% acetic acid in tap water, finally being hung up to dry in front of a gas or electric radiant.

Light green S.F. was first suggested by Dr. W. G. Dangerfield; I.C.I. Dyestuffs, Ltd., suggested that Lissamine violet 2R 124 (Victoria violet, colour index, 53) disulphine blue V.N. 150 (colour index, 712), Coomassie brilliant blue F.F.1 200 or crystal violet (colour index, 681) might also have the qualities required; that is, to dye proteins substantively from an aqueous acid bath, but to be immediately discharged from cellulose by dilute aqueous acid. It was found that the light green was the most effective of these dyes.

Recording.—In many cases, a simple inspection will provide the desired information, for example, in myelomatosis with increased γ-globulins, in nephrotic nephritis with an increase in α_2-globulin; gross increases in γ-globulins or decreases in albumin can also be recognized without further measurement. Nevertheless, there are some cases in which a nominally quantitative result will be demanded, for example, in the course of prolonged hepatitis. The apparatus we have used is indicated in Fig. 1; the image of an incandescent filament is formed on the paper strip just in front of a slit behind which is a photoelectric cell; the paper strip is traversed by hand across the slit, and the output from the photocell passed through a micrometer calibrated directly in extinction. The values of extinction are plotted against displacement along the paper.

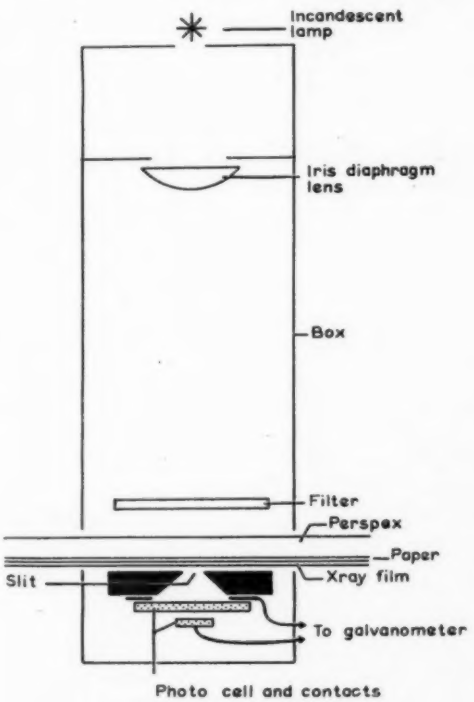

FIG. 1.—Scanning apparatus.

The area under the curve is divided into component curves which represent the component protein species. It is very difficult to estimate these components, and many methods have been recommended. We have drawn in by eye curves which appear to be symmetrical and whose areas summate to the total area. The γ-globulin curve alone is not symmetrical, and its faster moving boundary is determined by the slower boundary of the β-globulin. The areas are measured with a planimeter, converted to percentages of the whole and to concentrations in grams per 100 ml.

Results

The consistency of results was studied by performing five or more replicates on each of five sera. Table I shows the variability encountered;

TABLE I

RESULTS OF REPLICATES

Sample No.	Albumin	Globulins			
		α_1	α_2	β	γ
1	20·39	8·44	7·74	7·59	55·84
2	24·21	6·94	10·80	8·51	49·60
3	23·88	7·12	8·61	12·45	47·94
4	28·28	9·03	8·26	9·11	45·31
5	25·49	8·17	9·55	9·94	46·86
6	21·90	2·69	5·58	6·61	63·22
7	24·92	2·83	6·50	4·23	61·53
8	28·60	5·01	4·18	5·01	57·20
9	29·71	2·94	5·02	6·39	55·95
Mean	25·264	5·908	7·36	7·76	53·717

Samples 1 to 5 inclusive were analysed on 0.02 ml., 6 to 9 on 0.01 ml. The systematic error is clearly shown. All samples were from a single blood specimen taken from a patient with cirrhosis of the liver.

when the total quantity of serum applied to the paper is reduced, then the apparent concentration of the minor components is reduced, while that of the major components increases. If a constant amount of serum is used, the coefficient of variation ranges from 8% for a component which constitutes about 50% of the serum protein to 20% for one which constitutes 10%, and it is still greater for the lesser concentrations. These errors are very large, so large that it seems that changes in the minor components are not significant unless they are obvious to inspection of the strip by the eye; certainly only the albumin and γ-globulin estimates are sufficiently reproducible to warrant quantification.

Normal values are given in Table II; each value is the mean of duplicates.

TABLE II

ELECTROPHORETIC ESTIMATES OF SERUM PROTEINS IN TEN APPARENTLY HEALTHY SUBJECTS

Case No.		Albumin	Globulins				Total Protein
			α_1	α_2	β	γ	
M	1	3·08	0·40	0·67	1·09	1·78	7·0
	2	2·56	0·42	0·83	0·85	1·69	6·3
	3	2·67	0·50	0·66	1·21	2·36	7·4
	4	3·27	0·33	0·72	1·45	1·64	7·4
	5	3·17	0·32	0·62	1·18	1·72	7·0
	6	2·29	0·22	0·68	1·32	2·00	6·5
F	7	2·36	0·37	0·82	1·03	1·74	6·3
	8	2·67	0·29	0·67	1·13	1·94	6·7
	9	2·45	0·47	0·83	1·62	1·89	7·25
	10	2·53	0·45	1·07	1·32	2·14	7·5
Mean		2·705	0·377	0·757	1·22	1·89	
Standard deviation		0·3474	0·0877	0·1347	0·2192	0·22	

Fractions as g./100 ml.

Among pathological sera the narrow based, tall peak, usually in the γ-globulin, sometimes in the β-globulin band, of myelomatosis, is diagnostic, and can be recognized very easily; it often appears before the marrow aspirate is conclusive, and is sometimes present even when the total protein is normal or reduced. In nephrotic nephritis the α_2-globulin is increased, while the albumin and γ-globulin are reduced; these changes may appear in the absence of symptoms and when the only sign is proteinuria. The commonest abnormality is a rise in γ-globulin, often accompanied by a fall in albumin; it occurs in chronic infection, infective hepatitis and hepatic cirrhosis, many reticuloses such as Hodgkin's disease, sarcoidosis, some leukaemias, rheumatoid arthritis, lupus erythematosus, and other collagen diseases. These changes are quite non-specific and cannot be related to any specific disease process or organ—save that in renal disease there is usually a reduction, not an increase in γ-globulin. Since Kunkel's zinc sulphate turbidity test (1947) is claimed to provide a very reliable estimate of serum γ-globulin, we compared this test with our electrophoretic results.

A series of 45 sera was therefore investigated, duplicate estimates being made by both methods. The Kunkel reagent was prepared by dissolving 280 mg. barbitone and 210 mg. sodium barbitone in distilled water, adding 5 ml. 0.48% zinc sulphate heptahydrate, and diluting to 1,000 ml. The reagent was kept in an automatic burette protected by a soda-lime tube, because previous experiments had shown that serious *p*H changes occurred if it were kept in a bottle and dispensed with a pipette. To 6 ml. reagent was added 0.1 ml. serum, using a capillary pipette and a teat to prevent possible change of *p*H if expired air were to enter the fluid, and after more than 10 but less than 120 minutes, the opacity was measured in a "unicam" D.G. spectrophotometer at 650 mμ. The standard, to which is ascribed the value of 20 units, was prepared by placing 3 ml. 1.15% barium chloride dihydrate in a dry 100 ml. flask and diluting to the mark with 0.25N sulphuric acid; it is important to adhere strictly to these conditions. A series of standards was prepared and the average accepted as the extinction corresponding to 20 units.

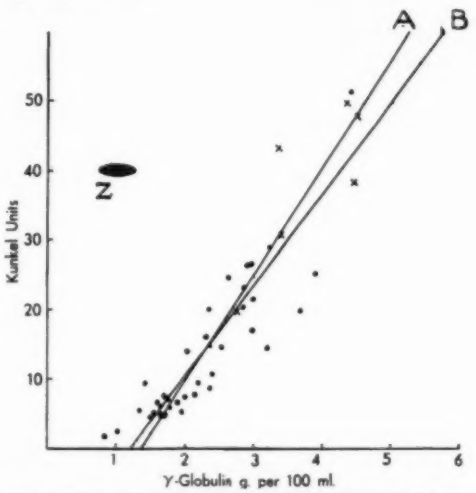

FIG. 2.—Scatter diagram of γ-globulin estimates and Kunkel's test. A, Regression line of X (dependent) on Y (independent). B, Regression line of Y (dependent) on X (independent). Z, Ellipse of error showing twice the standard error along each axis. Points marked with X are from patients with myelomatosis.

A scatter diagram of the results is shown in Fig. 2, together with the regression lines. The lines are described by

$$X = 1.384 + 0.0647Y \text{ (Line A)}$$
$$Y = 12.913X - 15.159 \text{ (Line B)}$$

where line A tells us the best estimate of X (the electrophoretic estimate of γ-globulin) given any value of Y (Kunkel units of opacity) and line B tells us the best estimate of Y given any value of X. The regression coefficient, r, is 0.914, based on 45 pairs of observations, indicating that about 83% of the variance is due to the relation between

X and Y, and but 16% to other sources of variation.

The experimental error was deduced for X and Y separately from the difference between each pair of observations, since a good estimate of the standard deviation between pairs is (for more than 20 pairs)

$$\sigma = \frac{\overline{w}}{1.13}$$

where $\overline{w}$ is the mean of the differences; and the standard error of any mean based on two observations is given by

$$\text{S.E.} = \frac{\tau}{\sqrt{2}}$$

We find that for the Ys, $\sigma = 0.5868$ and S.E. $= 0.415$ units; and for the Xs, $\sigma = 0.1894$ and S.E. $= 0.134$ units. As far as experimental error is concerned, the 95% confidence limits of any point on the scatter diagram can be illustrated by drawing an ellipse round each, using as semi-major axis 2×0.134 in the X direction and as semi-minor axis 2×0.415 in the Y direction. Such an ellipse is drawn on the scatter diagram, and it is obvious that the total error is somewhat greater than that which can be ascribed to lack of reproducibility of the analytical techniques. There is no evidence on the relation between the true γ-globulin level and the estimates which are made of it by our techniques, and it is valueless to speculate on this matter.

Comparison of the zinc sulphate opacity measurements in our own laboratory with those made elsewhere on the same sample of serum suggests that agreement between laboratories, though usually close, is not exact, and that there can be quite gross differences. For this reason we suggest that the regression curve we quote applies only to our own reagents, which give, on the 10 normal sera of Table II, a mean of 6.62 units with a standard deviation of ±1.1653; the best estimate of the 95% confidence limits (using the t test) is from 3.98 to 9.26 units, and for the 99% limits from 2.83 to 10.41 units.

Summary

The technique of filter-paper electrophoresis is laborious, and the precision of our version with aqueous staining not great.

It is useful particularly in the diagnosis of myelomatosis and of nephrotic nephritis.

The zinc sulphate turbidity test of Kunkel is simple and rapid; it correlates very closely with our estimation of γ-globulin, and is more suitable for studying the progress of a disease than is the more laborious electrophoresis.

We are indebted to Mr. F. Dewar for help in making our apparatus, and to Drs. J. Fielding, S. Shaw, and A. G. Signy for the gift of some sera.

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SIMPLIFIED ESTIMATION OF CALCIUM IN BIOLOGICAL MATERIALS BY FLAME PHOTOMETRY

BY

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Flame photometry has introduced rapidity, accuracy, and simplicity into the estimation of sodium and potassium in biological materials. When it is desired to prepare for the estimation by flame photometry of Ca^{++} in serum containing Na^{+} and K^{+} merely by diluting with distilled water, it must be kept in mind that for every 10 mg./100 ml. of Ca^{++} there is 325 mg./100 ml. of Na^{+}. Furthermore, the sodium spectrum lines are very near to, and more intense than, those of Ca^{++} so that Ca^{++} values obtained in this way are greater than actual values.

Following the wide use of flame photometry since 1945 (Barnes, Richardson, Berry, and Hood, 1945) many attempts have been made to estimate calcium; unsuccessful data remain unpublished, and hitherto reported methods are inaccurate or tedious.

In one of the earliest reports in this field Belke and Dierkesmann (1948) attempted the estimation using a flame photometer equipped with acetylene gas and a green filter reputedly not absorbing completely Na^{+} and K^{+} lights. Attempts were made to allow for Na^{+} and K^{+} interference by the addition of comparable amounts of these elements to the standard Ca^{++} solutions. The accuracy of this procedure is impaired by relative variability of Na^{+} and K^{+} levels in biological fluids and also by the fact that Ca^{++} *per se* is not measured.

Herrmann (1952) has described a method for the estimation of calcium, in which the main features are the recording of the green line in the Ca^{++} spectrum at 558 mμ, the use of acetylene gas for combustion, and a simple serum dilution of 1 in 5. Neither normal values for Ca^{++} in serum nor objective data are reported, but this method has been used by Bohnstedt, Herrmann, Baumann, and Füller (1953) to estimate the K^{+}/Ca^{++} serum ratio in normal subjects and eczematous patients. Isolated values for K^{+} and Ca^{++} are not given in this paper, and a remark is made that "bei unserer Methode die K/Ca-Quotienten stets relativ niedrige Werte ergaben." The value for Ca^{++} can, however, be determined theoretically from their data: for normal people the ratio is given as 1.54, and, if the accepted normal K^{+} serum value of 20 mg./100 is considered, the "normal" for Ca^{++} serum by this method is 13 mg./100. This value is high and agrees with values found by us when Ca^{++} was estimated merely by dilution of serum.

It has been realized for some time that this problem could be solved by precipitating Ca^{++}, thus removing interfering cations and carrying out the estimations on the redissolved and suitably diluted Ca^{++} sample.

The manufacturers of the Beckman spectrophotometer with flame attachment recognize (Application Data, DU-9-B) the necessity of precipitating Ca^{++} in Na^{+} and K^{+} mixtures in spite of all the advantages of this apparatus. Precipitated calcium oxalate is washed, centrifuged, and taken up in perchloric acid and isopropylalcohol. Explanations for the use of these two substances are not given. Acetylene gas is also used in this method.

While our investigations were being carried out Schlütz (1953) described a method based on the same principle of precipitation as calcium oxalate. No advantage is really gained with this method over the usual chemical methods, since three centrifugations and two washings are required. Furthermore, no details of the filter used are given and no objective data showing sensitivity and accuracy are reported. Acetylene gas was also used in this case.

Since the start of these investigations, the aim has been to establish a method to estimate the true amount of Ca^{++} in biological materials under the following conditions: (1) without Na^{+} and K^{+} interference; (2) saving time on the ordinary chemical methods, especially the two or more washings, re-centrifugations, and tedious titration; (3) adapted to a simple flame photometer, using coal-gas and compressed air instead of acetylene gas and additional oxygen.

An Evans "electroselenium" Ltd. flame photometer supplied with coal-gas and compressed air from a cylinder was used. Air pressure was adjusted at 10 lb./sq. in. (0.7 kg./cm.^{2}). The gas was regulated to produce 10 small cone-shaped flames at the top of the burner. Working with this empirical procedure, flame conditions usually change each time the burner is lit. This is obviated by interpolating with the unknown samples immediately higher and lower standards. Nevertheless, an absolute relationship, as shown below, between deviations in galvanometer scale-readings and concentrations of Ca^{++} exists. An interference filter with a peak at 620 mμ and additional special didymium glass to cut out interference by Na^+ was used.

With these conditions a whole deflexion of the galvanometer scale is reached with 30 p.p.m. of Ca^{++} when the potentiometer is adjusted to 1. Sometimes, however, due to variations in the supply or quality of gas, a complete deflexion is not reached with 30 p.p.m. For this reason, a 40 p.p.m. Ca^{++} solution is used as the strongest, and the potentiometer is adjusted between 1 and 2 until the whole deflexion is recorded in the scale of the galvanometer.

Preliminary Experiments

The first objective was to establish a linear relationship between deviations of the galvanometer scale-reading and the concentrations of Ca^{++} in a mixture containing no other cations: this simple relationship in a range from 0 to 40 p.p.m. is obvious from Fig. 1. Therefore, estimations of solutions containing only Ca^{++} do not represent any problem with this type of flame photometer.

The second objective was to measure the Ca^{++} content in samples of normal serum by simple dilution with distilled water. Values of about 13 mg./100 ml. were obtained by this procedure.

Finally it was necessary to determine the efficiency of the Ca^{++} filter in cutting out light due to Na^+ and K^+. Standard samples of Na^+ and K^+ were sprayed in the flame photometer. A Na^+ content of 8 p.p.m. (0.8 mg./100 ml.) and a K^+ content of 7.5 p.p.m. (0.75 mg./100 ml.) do not produce any deviation when the filter for Ca^{++} is tested. As, on the other hand, other trials showed (see below) that the Na^+ contained as impurity or residuum in the precipitated samples of Ca^{++} does not exceed this amount, this experiment assured that below that range all light due to Na^+ and K^+ was cut off.

Preparations of Standards

Calcium chloride prepared in the following way was used: AnalaR $CaCO_3$ is put in the oven overnight at 40° C. to dry it. Next day 12.5 g., which represents exactly 5,000 mg. of Ca, is weighed out. This is suspended with a small amount of distilled water in a volumetric flask and Na-free *N*-HCl is added (about 250 ml.) until it is completely dissolved. This is made

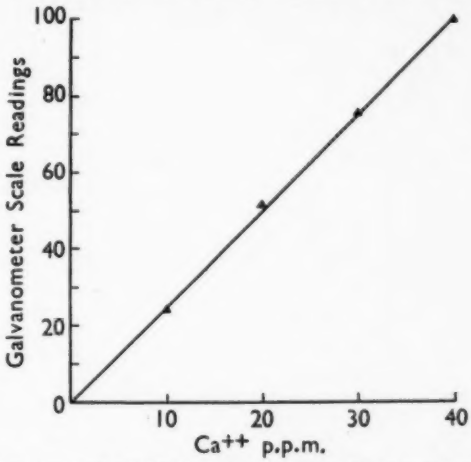

FIG. 1.—Linear relationship between galvanometer scale-readings and other cation-free Ca^{++} solutions.

up to 1.1 with distilled water. This solution is called the stock solution, and when kept in the refrigerator can be used indefinitely. From the stock solution the following standard solutions are prepared:

Solution	Stock Solution (ml.) Made up to 1,000 ml. H_2O	Ca^{++} Content in p.p.m.
A	2	10
B	4	20
C	6	30
D	8	40

Solutions used for recovery were either the same standard solutions or solutions prepared from stock solution.

Precipitation of Ca^{++} Samples

Samples containing 5 mg., 7.5 mg., 10 mg., 12.5 mg., and 15 mg./100 ml. of Ca^{++} were used. Two lots were made: (1) containing only Ca^{++}, and (2) Ca^{++} dissolved in a solution of NaCl, containing 305 mg./100 ml. of Na^+, a concentration similar to that of serum.

Two millilitres of each sample was taken, precipitated with ammonium oxalate, and allowed to stand for 30 minutes. Four millilitres distilled water was then added and the solution was centrifuged. The supernatant fluid was discarded, the precipitate dried and redissolved with 10 ml. 0.2 *N*-HCl.

Samples of the solutions containing only Ca^{++} were also estimated directly, i.e., without any manipulation. As a rule, values found after precipitation were lower than those found by direct estimation. Results are shown in Table I. From this experiment

TABLE I
RESULTS OF PRECIPITATING CALCIUM SAMPLES

Theoretical Values (mg./100 ml.)	Samples Estimated Directly by Flame Photometer	Samples Precipitated			Samples Precipitated Containing 305 mg./100 ml. Na^+				
		Ca^{++} Content	Na^+ Content (mg./100 ml.) as Impurity	Ca^{++} Content of Decanted Water	Ca^{++}	Na^+	Duplicate		Mean Ca^{++}
							Ca^{++}	Na^+	
5	4·9	4·6	0·5	0	5·6	2·25	5·1	1·2	5·3
7·5	7·5	5·8	0·5	0	7·4	2·0	7·1	1·2	7·2
10·0	10·0	9·1	0·49	0	10·0	2·6	8·8	1·2	9·4
12·5	12·5	11·06	0·62	0	10·8	1·5	10·8	1·1	10·8
15·0	15·0	12·5	0·42	0	12·7	2·4	12·3	1·1	12·5

Experiments with 2 ml. of the sample.

two points became clear: (1) The "loss" of Ca^{++} after oxalate precipitation does not follow any linear relationship (Fig. 2) but is a curve. As the Ca^{++} content in the decanted water of samples containing pure Ca^{++} (in samples to which Na^+ was added this experiment cannot be made because of the high Na^+ content) was zero, the apparent loss of Ca^{++} can only be due to interference. (2) Na^+ in a concentration similar to that of human serum does not produce any additional interference when Ca^{++} samples are simply precipitated without any washing.

Oxalic Acid Interference

When HCl is added to redissolve the precipitate, $CaCl_2$ is formed and oxalic acid is liberated. Moreover, some ammonium oxalate is still present as impurity, which is now converted to oxalic acid. As this substance remains in the solution analysed by flame photometry, we thought that oxalic acid was responsible for the interference. To prove this a simple experiment was done: 50 p.p.m. of oxalic acid was added to each standard and measured by flame photometry, these samples having been checked by oxalic acid-free standards. Results are expressed in Fig. 3 and confirmed our suspicion. The similarity of this curve with that shown in Fig. 2 is obvious. That oxalic acid produced a depressant effect on the Ca^{++} flame has not hitherto been reported, in spite of others (Application Data, DU–9–B; Schlütz, 1953) having tried to estimate Ca^{++} by flame photometry after similar precipitation. This effect was quite unexpected but not surprising, since Bills, McDonald, Niedermeier, and Schwartz (1949) have described a similar action of other organic compounds on Na^+ estimations. Oxalic acid can be destroyed by several oxidizing agents, but either some of these contain K^+ as a cation (it is obviously ridiculous to mix the precipitate with Na^+ or K^+, after it has been precipitated specifically to remove these cations) or else the added substance itself might produce a new interference effect. On the other hand, there is a very simple procedure to destroy the calcium oxalate molecule, i.e., to heat it at any temperature between 300° and 400° C. At this temperature it is converted to $CaCO_3$. This step has another advantage: the unknown sample is prepared in the same way as the standards, i.e., HCl is added to $CaCO_3$ to dissolve it.

Results of Precipitating Samples and Destroying Oxalic Acid

Samples containing 2.5 mg., 5 mg., 7.5 mg., and 10 mg./100 ml. of pure Ca^{++} were chosen to carry out this experiment. Different samples containing the same amount of Ca^{++} were estimated as above, and others after being left overnight in the furnace at 300°–400° C. All samples were carried out in duplicate. Results are shown in Table II. Complete agreement between expected and found results was obtained in the samples put in the furnace.

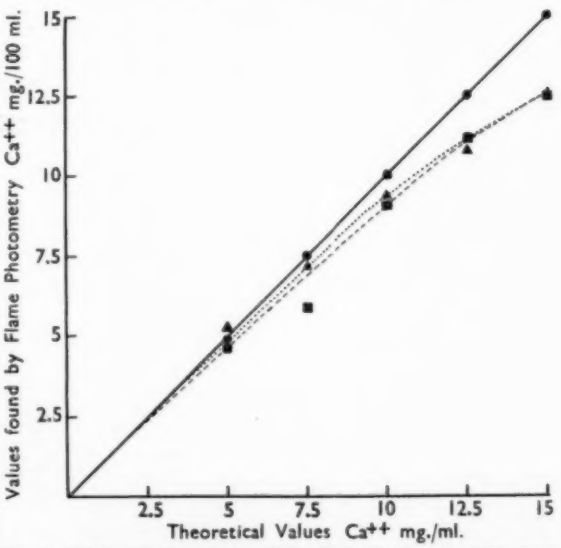

FIG. 2.—Relationship between theoretical values and those found by flame photometry. ● Non-precipitated samples. ▲ Na^+-free precipitated samples. ■ Samples precipitated containing 305 mg./100 ml. of Na^+.

TABLE II

RECOVERY OF CALCIUM IN DISTILLED WATER

Theoretical Values (mg./100 ml.)	Samples Estimated Directly by Flame Photometer	Samples Precipitated						
		Ca^{++} Content		Ca^{++} Mean	Na^{+} as Impurity (Mean)	Ca^{++} After 400° C. in Furnace		
			Duplicate				Duplicate	Mean
2·5	2·6	2·8	2·9	2·85	1·8	2·7	2·7	2·7
5·0	5·0	4·4	4·2	4·3	1·1	5·0	5·0	5·0
7·5	7·5	6·6	6·4	6·5	1·1	7·5	7·4	7·45
10·0	10·0	8·2	8·2	8·2	1·2	9·8	9·8	9·8

Experiments with 2 ml. of sample.

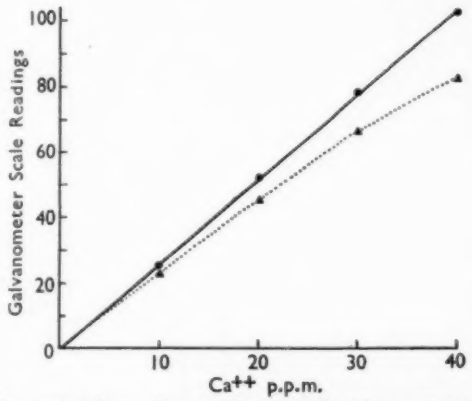

FIG. 3.—Interference effect of oxalic acid on Ca^{++} flame excitation. ● Samples diluted with distilled water only. ▲ Samples diluted with distilled water containing 50 p.p.m. of oxalic acid.

Experiments on Recovery of Ca^{++} in Serum

Since the method was working perfectly, it was decided to apply the technique to human serum. Serum was obtained from the same subject throughout and was diluted 3:2 (v/v) with distilled water. This dilution was used to economize in serum and to bring the Ca^{++} values to about 9 mg./100 ml., when different amounts of Ca^{++} were added. One mg., 2 mg., 3 mg., and 4 mg./100 ml. of Ca^{++} were added to different samples of this serum. All were done in duplicate. In this experiment only 1 ml. of the sample was taken in each tube, instead of 2 ml. used in the above experiments. The results are shown in Table III. Recovery of Ca^{++} was complete.

Several estimations in normal sera have since been made, and results ranging from 9.6 to 10.5 mg./100 ml. found. Values found in urine change with the metabolic status of the person as shown by chemical methods. It has also been applied to the estimations of Ca^{++} in cerebrospinal fluid, tap water, and milk.

Detailed Technique of Calcium Estimation by Flame Photometry

One ml. of sample is put in a 15 ml. conical "pyrex" centrifuge tube, perfectly clean and dry. One ml. of distilled water and 1 ml. of saturated (about 4 g./100 ml.) ammonium oxalate are added. They are mixed by rolling the tube between the palms of the hands and are left standing for 30 minutes. Then 4 ml. of distilled H_2O is added to "wash" free of Na^{+} and the sample mixed again. Samples are centrifuged at 2,500 r.p.m. for 10 to 15 minutes. Supernatant fluid is discarded by very careful decantation and tubes are left inverted on a sheet of filter paper for five minutes. Thus the last drops of liquid containing Na^{+} are absorbed. Tubes are placed overnight in a furnace adjusted at any temperature between 300° and 400° C. The precipitate is then dissolved in 5 ml. 0.2 *N*-HCl (Na^{+}-free). Dissolution is favoured by putting the tubes for two minutes in a boiling water-bath. Samples are diluted 1:1 (this is the proper dilution for serum; greater dilutions may be required by urine or other biological samples), transferred to small beakers, and the actual Ca^{++} content measured by flame photometry, interpolating the reading of the unknown sample between immediately lower and higher standards.

Ca^{++} solutions containing 10, 20, 30, and 40 p.p.m. are used as standards. The method of prepar-

TABLE III

RECOVERY OF CALCIUM IN SERUM

Samples	Ca^{++} Added (mg./100 ml.)	Theoretical Values (mg./100 ml.)	Ca^{++} Content			Na^{+} Content (Mean) (mg./100)	K^{+} Content
				Duplicate	Mean		
Diluted serum ..	—	—	7·0	7·0	7·0	4·0	0
" " ..	1	8·0	8·0	8·0	8·0	4·9	0
" " ..	2	9·0	9·0	9·0	9·0	4·0	0
" " ..	3	10·0	10·0	10·0	10·0	3·9	0
" " ..	4	11·0	10·9	10·9	10·9	4·5	0

Experiments with 1 ml. of sample. Initial Na^{+} content of diluted serum, 233·0 ml./100 ml. Initial K^{+} content of diluted serum, 10·15 mg./100 ml.

ation has been described above. As a routine we make up beforehand the dilutions of all the samples to be analysed in one session.

The procedure to make the Ca^{++} estimations of samples is the following:

Once the air pressure has been adjusted at 10 lb./sq. in. and the gas supply has been regulated according to the empirical procedure described above, the 0 and the 100 are checked in the galvanometer scale-reading. Distilled water is sprayed in the flame photometer and the galvanometer is adjusted at 0. Then the standard solution D (40 p.p.m. of Ca^{++}) is sprayed in the flame photometer and the potentiometer is adjusted between divisions 1 and 2 until a whole deflexion (that is, 100 divisions) is recorded in the galvanometer. The other standard solutions A, B, and C (10, 20, and 30 p.p.m. of Ca^{++} respectively) are now sprayed and the galvanometer deviations are recorded. Thus, when an unknown solution is estimated, it is possible to know between which two standards it lies. The unknown sample is now sprayed and the galvanometer reading recorded. The standard solution which produced deviations immediately lower and higher than this sample are checked again and the result is calculated by interpolation.

An example illustrates this procedure: a serum sample diluted finally at 1 in 10 for flame photometry estimation gives a deviation of 26 in the galvanometer dial. This lies between standards A and B, which on re-checking now give deviations of 25 and 52 respectively. The unknown sample contains obviously all the Ca^{++} of standard A plus an extra amount. This latter amount is calculated by means of the following formula: $\frac{D}{C} = \frac{d}{x}$ where D = difference of galvanometer deviations between the two standard solutions, C = difference of Ca^{++} content between these standard solutions, d = difference of galvanometer deviations between the unknown sample and standard solution immediately below it, x = Ca^{++} content of difference d. In the present instance, the equation would be

$$\frac{52-25}{10} = \frac{26-25}{x} \text{ and } x = \frac{10}{27} = 0.3$$

Thus, 0.3 is the extra amount which must be added to 10 p.p.m. (Ca^{++} content of standard A). Therefore, the Ca^{++} content of the diluted analysed sample will be 10+0.3=10.3 p.p.m., and as it is a 1 in 10 dilution the Ca^{++} value of the serum will be 10.3 $\times$10=103 p.p.m., or converting p.p.m. to mg./100 ml., it will be 10.3 mg./100 ml.

When a great number of estimations have to be carried out, it is convenient to construct a table, from which the calculated values may be obtained directly.

Occasionally when analysing urine, milk, or other biological materials with high Ca^{++} content, a solution of 1 in 10 contains still too much Ca^{++} and the galvanometer deviation reaches more than a whole deflexion. In this case it is necessary to dilute again the sample until the figure recorded in the dial is less than 100. This dilution is a factor by which the Ca^{++} content of the sample sprayed in the flame photometer must be multiplied in order to obtain the real Ca^{++} content of the original sample.

When calculating the results in the case of Ca^{++} serum estimations attention must be drawn to the fact that the initial volume of the serum sample was 1 ml., the volume when redissolved in HCl was 5 ml. This solution was then diluted by a factor of 2 to make it suitable for flame photometer estimation. Therefore, in the case of serum, the value obtained by analysing the diluted sample must be multiplied by 10.

In Table III values of Na^{+} apply to non-diluted samples, i.e., those solutions analysed by flame photometry contained 1 in 10 of the values given; therefore, all values of Na^{+} are below 0.8 mg./100 ml., that is completely cut off by the Ca^{++} filter.

Although the step of converting oxalate to carbonate by leaving the tubes in the furnace is very simple, it requires a thermostat, which may not be readily available in all laboratories. To avoid this possible hindrance it was decided to make further experiments using a simple sand-bath. Fine sand was placed in a tin tray and heated by one or more Bunsen burners, a thermometer being placed in the sand to record temperatures. It was observed that once the required temperature (about 360° C.) was reached, it could be readily controlled within 10° C. by slightly adjusting the burner(s). When half the length of the tubes containing the calcium oxalate precipitate was buried in the sand and left for 30 minutes at 360° C. the complete conversion of oxalate to carbonate was achieved and the values of Ca^{++} recovery were the same as those obtained by putting the samples in the furnace.

Certain precautions, however, must be taken. (1) The precipitate at the bottom of the tube must be dry before placing it in the sand. (2) When several series of estimations have to be carried out time can be saved by placing the tubes in the sand-bath at 200° C. When the temperature reaches 360°, the conversion of oxalate is completed in 30 minutes. (3) Care must be taken that the sand-bath is uniformly heated.

Conclusion

This is a combined chemico-physical method that makes feasible and easy the estimation of the true amounts of Ca^{++} in biological samples even when mixed with Na^{+}, K^{+}, and other biological substances, such as proteins, etc. The advantages over usual chemical methods can be summarized as follows:

It avoids the repeated washings and the re-centrifugations.

It suppresses the tedious step of titration, substituting an estimation on the flame photometer that takes 10 seconds. For this reason it is an extremely useful method when a large number of Ca^{++} estimations must be made.

Complete accuracy is reflected in the objective data given. The slightly high results that are often

obtained by manganometry, due to the presence of reducing substances, are obviated by this method.

Flame photometry for Na^+ and K^+ estimations is becoming a common method in this country and the same apparatus can be used for Ca^{++} estimations with the simple addition of the filter for Ca^{++}.

Summary

A method is described in detail for estimating Ca^{++} when mixed with other cations in biological materials, using a commonly available type of flame photometer, burning simple coal-gas and equipped with an interference filter.

An absolutely linear relationship between galvanometer deviations and Ca^{++} concentrations ranging from 0 to 40 p.p.m. is shown.

Na^+ and K^+ concentrations which can be admitted as "impurities" without affecting Ca^{++} estimation are discussed.

An interference effect of oxalic acid on Ca^{++} flame excitation is described for the first time. This is overcome by heating the samples at 300°–400° C., which destroys oxalic anion.

The complete recovery of Ca^{++} in distilled water and in human serum is shown in tables.

Advantages of the present method over routine chemical methods are that it avoids the repeated washings and subsequent recentrifugations and the tedious titration, and gives greater accuracy.

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A STUDY OF THE BLOOD PYRUVIC ACID LEVEL IN PATIENTS WITH CONGESTIVE HEART FAILURE

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The studies of the biochemical changes in heart failure are as interesting and important as those in the field of haemodynamics. The metabolic changes are not only the results of disturbed metabolism in circulatory or cardiac failure but in themselves may become influencing factors in failure.

Among the products of metabolism, pyruvic acid is of great importance because it is an intermediary in carbohydrate, as well as in protein and in fat metabolism. Since newer methods for its estimation have been introduced by Lu (1939) and by Friedemann and Haugen (1943), several authors have studied the role of pyruvic acid in heart diseases (Altschule and Rosenfeld, 1947 ; Barenghi, Scarlotti, and Scafidi, 1941 ; Lasch, 1952 ; Torre, Teece, and Baldantoni, 1950 ; Yanof, 1942), and a survey of the changes in pyruvic acid levels in other diseases and in relation to therapy has been published by Markees (1951a).

The purpose of this investigation was to study the level of blood pyruvic acid in patients with congestive heart failure and to ascertain: (1) whether the degree of severity of failure was reflected in changes of blood pyruvic acid values ; (2) whether any relationship existed between liver size, liver function, and blood pyruvic acid ; (3) whether therapy would influence the blood pyruvic acid level.

Clinical Data

Ten healthy subjects fasting and at rest were chosen to obtain normal values of blood pyruvic acid. Forty patients, 20 men and 20 women, in various stages of congestive heart failure were also selected. The cardiac cases, classified according to aetiology, had arteriosclerotic or hypertensive cardiac and vascular diseases (20 cases) ; rheumatic heart disease (15 cases) ; chronic cor pulmonale (five cases).

Classified according to clinical severity, five patients had on admission no apparent signs of decompensation ; 17 patients suffered from mild or moderate failure ; 14 were in severe failure ; and four were in very severe failure.

The patients were on a sodium-free diet. Most of them had an intake of 1,500 to 2,000 calories and in addition received 50 mg. vitamin B_1. Treatment consisted of digitalis, cardophyllin, mercurial diuretics, and dicoumarol adapted to the needs of each patient.

The effect of co-carboxylase on the blood pyruvic acid level was studied in 13 patients. These patients were either given one single injection, or consecutive daily injections of 50 or 100 mg. intravenously.

Methods

For the determination of the blood pyruvic acid level Lu's hydrazin method as modified by Friedemann and Haugen (1943) was used, and Markees' (1951b) technique of boiling the trichloracetic acid filtrate for five minutes to destroy aceto-acetic acid. Blood was always taken in the morning while the patient was fasting and in bed. In 10 patients a second estimation was done one or two hours after the first sample, with the patient still fasting. In half of the cases further serial pyruvic acid samples were taken, as many as 30 in one patient. Blood sugar, cholesterol fractions, serum albumin and globulin, cephalin-flocculation, thymol turbidity, and the Weltmann tests were also determined.

Results

The results are illustrated by Figs. 1 and 2 and Table I.

The blood pyruvic acid values in 10 healthy individuals varied between 0.53 and 1.08 mg.%.

Cardiac patients with no heart failure or with mild failure had normal pyruvic acid values. Eighty per cent. of the values in moderate failure and nearly half the values in severe failure, not complicated by acute events, were also normal.

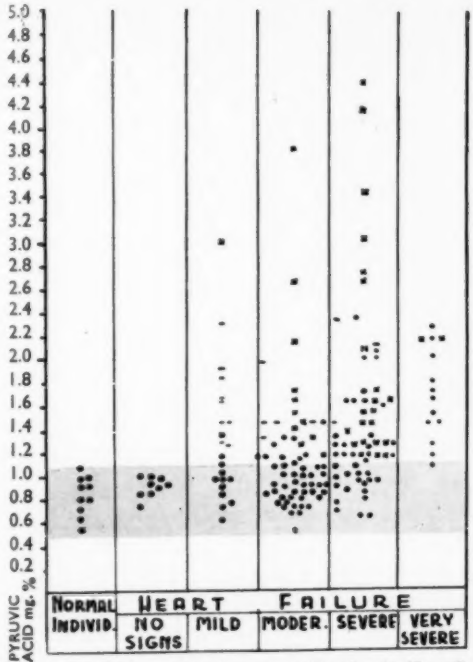

FIG. 1.—Distribution of blood pyruvic acid values in 40 cardiac patients and in 10 normal individuals. Crosses and dashes indicate values during or following severe complications. Shaded area indicates the range of normal values.

The remainder were slightly to moderately elevated (up to 2.3 mg.%), as were all the values in the very severe cases. Especially high pyruvic acid levels (up to 4.4 mg.%) were noted during and following severe complications, such as acute myocardial infarction, pulmonary oedema, hypertensive encephalopathy (crosses in Fig. 1 and Table I). Such hyperpyruvicaemia during acute episodes was found even in patients with mild failure (see Fig. 2). The pyruvic acid level showed a tendency to drop with clinical improvement, and to rise with the increase of the failure (see Table I).

There was no clear correlation between elevation of the blood pyruvic acid level and the extent of the liver damage, as indicated by the above-mentioned tests.

High pyruvic acid levels were associated with elevated blood sugar values in over half the instances. On the other hand, high blood sugar values (in the non-diabetic patients) almost always coincided with an increase in pyruvic acid concentration in the blood.

Cases No. 24 and 28 will be described in more detail.

Case Reports

Case 24.—A. Z., a man aged 52, suffered from arterio-sclerotic heart and vascular disease, and recurrent myocardial infarction.

He was admitted to the hospital on December 5, 1951, four days after a recent infarction. He had moderate heart failure, and a slightly enlarged liver. Liver function tests were normal except for an increased urine urobilinogen excretion. Blood pyruvic acid values were high. There was rapid clinical improvement and a drop in the blood pyruvic acid level, remaining low during the following week. Subsequently there was a slow but steady rise in the blood pyruvic acid in spite of the patient feeling well, but suddenly the blood pyruvic acid level rose accompanied by an elevation in blood sugar after occasional anginal pains.

On January 10, 1952, the patient experienced sudden, transient shock with signs of fresh coronary infarction. The blood pyruvic acid level was very high accompanied once more by a rise in the blood sugar and the disappearance of blood eosinophils the following day. The heart failure increased in severity with further enlargement of the liver and scleral jaundice. During the next few days there was a temporary clinical improvement with a slight drop in the blood pyruvic acid and sugar levels. The patient died after a further acute event, probably a fresh

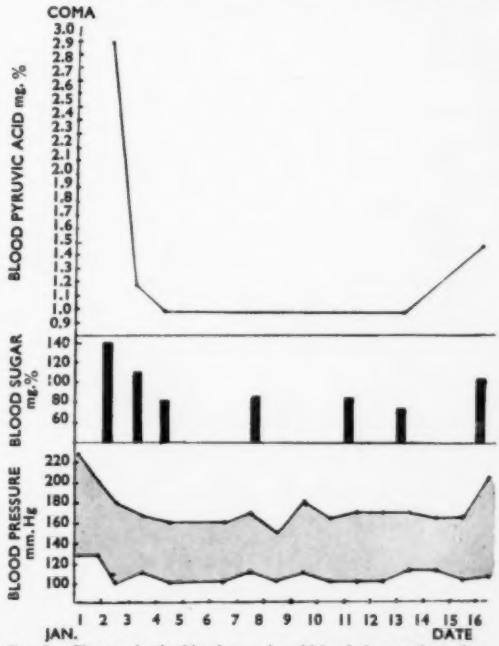

FIG. 2.—Changes in the blood pyruvic acid levels in a patient after a short episode of hypertensive encephalopathy (Case 28). Note the parallel blood sugar values.

TABLE I

INITIAL, MAXIMAL, AND MINIMAL BLOOD PYRUVIC ACID VALUES IN 40 CASES OF CARDIAC DISEASE RELATED TO SEVERITY OF HEART FAILURE AND TO CERTAIN BIOCHEMICAL TESTS

Case No.	Primary Disease	No. of P.A. Determinations	Initial Values						Maximal Blood Pyruvic Acid Values		Minimal Blood Pyruvic Acid Values	
			Severity of Heart Failure	Enlargement of Liver	Liver Function Tests	Urine Urobilinogen	Blood Sugar (mg.%)	Pyruvic Acid Level (mg.%)	Severity of Heart Failure	Pyruvic Acid (mg.%)	Severity of Heart Failure	Pyruvic Acid (mg.%)
	Arteriosclerotic Heart and/or Vascular Disease											
1	Myocardial infarction, healing, mild diabetes	1	Mild	No signs	Neg.	Normal	156	1·1				
6	Rheumatic heart disease inactive, mild diabetes	2	Severe	++	"	"	236	1·17			Moderate	0·85
9	Myocardial infarct, healing	1	Mild	No signs	—	"	89	0·73				
11	Old myocardial infarct	1	Moderate	++	Neg.	"	82	0·8				
12		2	Severe	+++	"	—	94	1·12	Severe	1·12	"	0·8
17	Mild diabetes	9	"	++++	±	Increased	118	1·72	"	2·90	"	0·68
19	Angina, old myocardial infarction	1	Mild	+	Neg.	Normal	105	1·02				
22	Chronic bronchitis, cor pulmonale	4	Severe	++++	"	Increased	78	1·08	"	2·0?	"	0·67
24	Acute myocardial infarction	30	Moderate	+	±	"	87	2·18	"	4·4†	"	1·0 (Died)
26	" " "	14	Severe	++++	±	Normal?	138	1·18†	Very severe	2·17†	Severe	0·9
27	Old " "	2	No signs	No signs	Neg.	"	84	0·9	No signs	0·99		
30	" " "	2	Mild	" "	"	—	—	0·6	Mild	0·91		
31	Mild diabetes	4	Severe	++	"	Normal	236	1·55†	Severe	1·55†	Moderate	0·99
36	" "	1	"	+++	"	Increased	148	1·18				
39	Urinary infection	1	Moderate	+++	±	"	113	0·9				
	Rheumatic Heart Disease											
2	Inactive	2	Moderate	+++	Neg.	Normal	100	0·9; 1·04*				
3	"	2	Very severe	+++++	"	"	105	1·66; 1·54*				
4	Inactive, subacute bacterial endocarditis	3	No signs	+	"	"	113	0·99	No signs	0·99	No signs	0·83
7	Inactive	7	Moderate	+++	Neg.	Increased	89	0·85	Moderate	1·12	Moderate	0·7
8	Inactive, cor pulmonale	10	Severe	+++	±	"	99	1·17	Severe	1·17	"	0·8
10	Inactive, hypertensive cardiovascular disease, chronic cholecystitis	3	Moderate	++	Neg.	Normal	82	0·73	Moderate	0·73	"	0·63
13	Inactive, cardiac cirrhosis	3	Severe	++++	+	—	83	0·63	Severe	0·8	Severe	0·63
16	Inactive	8	Moderate	++	Neg.	Normal	87	1·32	"	1·32	Moderate-mild	1·08
18	"	4	"	+++	±	Increased	108	1·00	"	1·45†	Moderate	1·0
21	"	8	Very severe	++++	±	"	144	2·17	Very severe	2·28	Very severe	0·99 (Died)
25	Active	9	"	+++	Neg.	"	136	1·45	" "	2·0	Moderate-severe	0·83
29	?Active, post partum	11	Severe	+	"	Normal	71	1·26	Severe	1·55†	Moderate	0·99
32	Active	7	Mild	No sign	"	"	102	0·83	Moderate	1·45	Mild	0·83
33	Inactive, arterial embolism, ischaemic neuritis	2	Moderate	+	±	"	139	3·83†	"	3·83†	Moderate	1·26
40	?Active, 7th m. pregnancy	15	Severe	++++	Neg.	"	85	1·0	Severe	1·45†	"	0·75
	Hypertensive Cardiovascular Disease											
5	Hypertensive cardiovascular disease	1	No sign	No sign	Neg.	Normal	82	0·91				
14	"	1	"	None	"	"	83	0·72				
28	Hypertiensive cardiovascular disease, arterio-sclerotic vascular disease, encephalopathy	16	Mild	No sign	"	"	142	3·0†	Mild	3·0†	Mild	0·99
35	Hypertensive cardiovascular disease	9	Severe	++	"	"	80	1·20	Severe	1·63	Moderate	1·08
37	"	1	Moderate	±	"	—	100	1·36				
38	" " "	1	No sign	None	"	Normal	95	0·83				
	Other Heart Disease											
15	Cor pulmonale, chronic bronchitis and asthma	2	Severe	+++	Neg.	Normal	106	0·85			Severe to moderate	0·68
20	Chronic asthma and bronchitis, cor pulmonale	2	"	+++	—	"	—	0·9			Moderate	0·5
23	Chronic bronchitis, cor pulmonale	2	Very severe	++++	Neg.	Increased	65	1·17; 1·08*				

* Determinations on two consecutive days. † After acute episodes.

myocardial infarction, with a new rise of blood pyruvic acid to a very high level several hours before the clinical signs of the new attack. Co-carboxylase decreased the pyruvic acid level for a short time without signs of clinical improvement.

Case 28 (Fig. 2).—B. S., a man aged 46, had hypertensive cardiovascular disease, an old myocardial infarction, and residual hemianopia following a recent cerebral event. He was in mild heart failure. The blood pressure was usually 180–200/100–110 mm. Hg.

He was admitted to hospital on January 1, 1952, in a state of unconsciousness which had developed suddenly after a rise in blood pressure to 225/150 mm. Hg. He recovered consciousness within a few hours of admission. He was exhausted and vomiting, but no pathological change was seen in the neurological or other clinical findings other than the old hemianopia. Next morning the patient had a high fasting blood pyruvic acid level, and the blood sugar level was elevated with transient glycosuria. Liver tests were negative. Rapid clinical improvement on the first day was maintained during the following days, the pyruvic acid and sugar dropping to normal; the blood pressure was moderately raised. On January 16 he was again "feeling bad," and the blood pressure and blood pyruvic acid and sugar levels had all risen. No change in the degree of heart failure was discernible. Within one more day recovery was complete.

Discussion

Technique.—The hydrazin method of Lu and of Friedemann–Haugen has been challenged by several authors (Elgart and Nelson, 1941; Klein, 1941; Lestradet and Guest, 1951), as it is an estimation of keto-acids in general and not only of pyruvic acid. Markees (1951b) has shown that boiling the filtrate for five minutes destroys the aceto-acetic acid (the main competitor of pyruvic acid) without loss of pyruvic acid.

Normal Values.—The normal values for blood pyruvic acid in healthy persons obtained in this study (0.53–1.08 mg.%) are in conformity with other reports irrespective of the methods used (Bueding, Wortis, and Stern, 1942; Friedemann and Haugen, 1943; Lu, 1939; Markees, Käser, and Lanz, 1950).

Relation between Degree of Failure and Pyruvic Acid Level.—Fig. 1 shows clearly that patients with heart disease, but without any clinical signs of failure, and patients with only mild symptoms of failure have normal blood pyruvic acid values. This fact is to be expected and it is likewise conceivable that very severe failure could raise the blood pyruvic acid as a sign of disturbed metabolism. On the other hand, several moderately severely and even very severely ill patients showed throughout the weeks of observation an almost normal blood pyruvic acid level. Both results are in agreement with the reports of other authors (Barenghi *et al.*, 1941; Torre *et al.*, 1950; Yanof, 1942). Even a very high blood pyruvic acid value, however, does not necessarily mean a fatal outcome, as was shown in two patients with a pyruvic acid level of 3 and of 4 mg.%. Their recovery was accompanied by a return of the pyruvic acid level to almost normal figures.

When the pyruvic acid determinations revealed nearly normal figures despite the moderate or severe state of failure an improvement of the patient's condition sometimes decreased slightly the values from 1.0 or 0.9 to 0.8 or 0.7. Improvement of failure is generally followed by a drop in abnormally high blood pyruvic acid values, whereas a deterioration mostly caused a rise in the pyruvic acid level. As a rule, sudden complications elevated the blood pyruvic acid higher and more rapidly than a slow deterioration in a prolonged chronic illness.

It is of interest that in cases No. 8, 28, 29, 34, and 40 the elevation of the pyruvic acid level preceded by several hours and even for a day or two the appearance of clinical signs of deterioration. Should this finding be confirmed in a greater number of cases, it might not only prove valuable as a prognostic sign, but it might also demand from the physician greater vigilance when it occurs in a chronically ill patient with apparently innocuous complaints.

Relation between Liver Function Tests and Blood Pyruvic Acid Level

Severe liver damage may cause a rise in the blood pyruvic acid level (Davis and Bauer, 1944; Marche and Marnay, 1946; Amatuzio and Nesbitt, 1950; Kleeberg and Gitelson, 1953) because the damaged organ cannot metabolize this acid. On the other hand, severe heart failure is not infrequently associated with liver damage as a result of prolonged stasis and anoxia (Lichtman, 1949). It is therefore conceivable that liver insufficiency may be responsible, at least partly, for the rise in pyruvic acid level in severe heart failure (Amatuzio and Nesbitt, 1950).

In several of the severely ill patients a marked rise in pyruvic acid level coincided with deterioration in liver function (not indicated in Table I). However, in general there was no close correlation between the changes in pyruvic acid level and in the results of the liver tests employed. This may be partly explained by the fact that these tests do not represent all facets of the manifold functions of the liver (King, 1952; Popper, 1952; Staub, 1950).

* Determinations on two consecutive days. † After acute episodes.

Influence of Therapy.—Since only the severe or very severe cases of failure had an elevated blood pyruvic acid level the entire well-established therapeutic regime had to be instituted without taking the risk of evaluating one procedure alone. Thus we can only state that general therapy lowers high pyruvic acid values if the clinical picture improves. On the other hand, if the severity of the circulatory failure persists, with or without treatment, the high pyruvic acid figures remain unaltered.

In 13 patients an additional therapeutic trial was made with a phosphorylated thiaminchloride (co-carboxylase) intravenously.* Williams, Egana, Robinson, Asper, and Dutoit (1942) reported that intravenous injection of di-phospho-thiamine suppressed the high pyruvic acid level in thyrotoxic patients. In a series of animal experiments, as well as in clinical studies on diabetics and on pregnant women, Markees has urged the therapeutic use of co-carboxylase. His results (Markees and Meyer, 1949) were remarkable in alloxan-diabetic rabbits as well as in human beings with severe diabetes. The effect was especially striking in regulating acidosis.

Using this method, Torre *et al.* (1950) observed in cardiac patients with an elevated pyruvic acid level a drop of almost 29% after the intravenous administration of co-carboxylase, whereas the injection of 100 mg. vitamin B_1 alone depressed the level by only 6%. Lasch (1952) noted a significant therapeutic advantage with this phosphorylated thiamin in children with toxic diphtheria, who, of course, at the same time received specific therapy.

Our experiences with this substance are that in a few cases with an initially high blood pyruvic acid level the levels were reduced one or two hours after a single intravenous injection of 0.05 co-carboxylase. In some other patients with a pyruvic acid level at the upper border of normal, no such effect was seen. In three severely ill patients with an initially high blood pyruvic acid level a daily administration of 0.05 or 0.1 co-carboxylase caused a prompt decrease to nearly normal figures, associated with a mild clinical improvement. In two other patients, clinical improvement and a drop in the blood pyruvic avid level were obvious after the co-carboxylase therapy. One extremely ill patient died, however, in spite of a return to normal of the blood pyruvic acid level. In five cases after a single injection of 0.05 co-carboxylase or after repeated injections on alternate days no unequivocal effect on the blood pyruvic acid level was observed when the blood was examined 24 hours after the injection.

Probable Mechanism of Pyruvic Acid Changes in Congestive Heart Failure.—There is no facile explanation for fluctuations in the blood pyruvic acid level in cases of congestive heart failure. The most plausible explanation would be that anoxaemia of tissues causes this intermediate metabolic substance to accumulate (Ochoa, 1939 ; Torre *et al.*, 1950 ; Yanof, 1942).

It is well known that anoxia increases the breakdown of glycogen and decreases the oxidation of its split-products, pyruvic acid and lactic acid, in the liver. This leads to a rise in the concentration of these products, as well as of glucose, in the blood.

Actually several of the patients with severe heart failure or myocardial infarction had simultaneously high blood pyruvic acid and high sugar values.

However, our studies, as well as those by others, show that many patients with moderate or even more severe signs of heart failure still have an almost normal blood pyruvic acid level. This was particularly evident in patients with chronic cor pulmonale and long-standing failure. Rasmussen and Storstein (1951) found almost normal arterial oxygen saturation in many patients with chronic heart failure and dark-blue cyanosis. It is therefore possible that some of the chronically ill patients, even those in severe failure, did not have marked anoxaemia. On the other hand, Amatuzio and Nesbitt (1950) found in patients with pulmonary emphysema and marked hypoxia almost normal pyruvic acid values. This seems to indicate that hypoxia by itself, unless very severe, is not sufficient to cause hyperpyruvicaemia.

As another explanation of hyperpyruvicaemia after myocardial infarction Altschule and Rosenfeld (1947) point to the possibility of increased catabolism as part of a general stress reaction (Selye, 1950).

Summary and Conclusions

Forty patients with congestive heart failure of various grades were examined for blood pyruvic acid by means of the Lu–Friedemann–Haugen method. All patients were on bed rest and a standard sodium-free diet supplemented with at least 50 mg. vitamin B_1 per day.

Our normal figures for blood pyruvic acid in 10 healthy individuals conformed with the values found by other authors, by means of the same or by other methods of determination, with values ranging from 0.53 to 1.08 mg.%.

* Supplied through the courtesy of Hoffmann-LaRoche, Basle.

The majority of the severe and all very severe cases had high blood pyruvic acid values. Every deterioration, especially acute, was accompanied by a rise in pyruvic acid. Improvement of the clinical picture was paralleled by a decrease of blood pyruvic acid, often to normal figures. Even high values, 3 or 4 mg.%, need not necessarily indicate a fatal outcome.

One blood pyruvic acid determination in cases of congestive heart failure is only of minor value because mildly or moderately ill patients and some severely ill patients showed normal or almost normal values; chronic cases may do so for a considerable length of time in spite of severe heart failure.

In several patients, in whom serial blood pyruvic acid determinations were carried out, an elevation of pyruvic acid to above normal preceded the appearance of clinical symptoms of deterioration or of complications. It would appear, therefore, that repeated pyruvic acid tests might be of prognostic value if associated with other clinical and biochemical findings.

There was no significant relation between the blood pyruvic acid level and the results of some of the so-called liver tests. A definite connexion could be evaluated in certain cases of heart failure between blood sugar and pyruvic acid values.

In 13 cases thiaminpyrophosphate was administered intravenously to patients who at the same time received the usual cardiac therapy. In cases receiving repeated injections the elevated blood pyruvic acid dropped to nearly normal, but was not uniformly accompanied by marked clinical improvement.

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MALIGNANT CELLS IN CEREBROSPINAL FLUID

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Ever since the beginning of the present century, the identification of the cells in cerebrospinal fluid (C.S.F.) has been recognized as an important diagnostic procedure. Unfortunately, it has always been found a difficult matter to make stained films of these cells, and in fact the preparation of Romanowsky-stained, air-dried films comparable with those of blood has been considered almost impossible (Reichmann, 1911). In spite of the introduction of several alternative techniques, none has found a permanent place in laboratory practice, and the preparation of a film is usually omitted.

Presumably as a result of this difficulty, the number of reported cases in which tumour cells have been detected in C.S.F. is small. The fact that they can be found at all does not seem to be generally known. The purpose of this paper is to record seven cases of which six were subsequently proved to have meningeal spread of a malignant growth. (No post-mortem examination was permitted on the seventh.) These cases illustrate the importance of making stained films in cases of cerebrospinal pleocytosis, and a method for preparing such films is described.

The Previous Literature

Tables I and II list the 66 more or less acceptable cases which I have been able to find in the literature. In many of them the descriptions are inadequate, but no case has been excluded on this account alone.

Few of the reported cases are adequately illustrated, and it is difficult to draw from them a reliable picture of the types of cell to be expected from different tumours. One striking feature of the two tables is the high proportion of cases in which the meningeal infiltration was secondary to a carcinoma outside the central nervous system. A probable explanation of this is that metastatic carcinoma cells are relatively large, and are therefore likely to be recognized in the counting-chamber. The smaller cells of, say, a medulloblastoma are likely to be passed over as lymphocytes or as "mononuclear cells."

Cases Excluded from the Tables.—The cases of Mohr (1912), Emanuel (1927), and Thurel, Poujol, and Contamin (1949) have been excluded because of unconvincing cytological data and the absence of any evidence of meningeal spread of the growths. The first 11 of Forster's (1930) 12 cases are excluded for the same reasons; the illustrations and descriptions are not in accord with my own experience of tumour cells nor with that of other authors, and in several cases the tumours were benign.

TABLE I

PRIMARY INTRACRANIAL AND INTRASPINAL TUMOURS

Author	Type of Growth and Remarks
Dufour (1904)	Cauda equina tumour. Doubtful interpretation of large cells in C.S.F.
Rindfleisch (1904)	Tumour of third ventricle
Léri and Catola (1905)	"Epithelioma" of lumbar cord, thought to be of ependymal origin
Loeper and Crouzon (1906)	"Sarcoma" of occipital cortex (drawings)
Sicard and Gy (1908)	Sarcoma of dura mater (drawing)
Guillain and Verdun (1911) and Verdun (1912)	Ependymal glioma of corpus callosum (drawings)
Fischer (1922, Part II, Case 1)	Multiple tumours of brain and cord, ? type
„ („ „ „ „ 2)	Melanosarcoma of brain
„ („ „ „ „ 3)	Glioma
„ („ „ „ „ 4)	Unproved spinal tumour
„ („ „ „ „ 5)	Sarcoma of spinal dura
Cairns and Russell (1931)	Pituitary adenoma
Ostertag (1932 and 1933)	Spongioblastoma of cerebellum (photographs)
Walt (1939)	Medulloblastoma
Schroeder *et al.* (1945, 2nd case)	Glioma of cerebellum (photograph)
Platt (1951, Case 5)	Glioblastoma multiforme (ventricular aspiration; no evidence of meningeal spread) (photograph)
Platt (1951, Case 6)	Medulloblastoma (cisternal fluid; photograph)
„ („ „ 7)	? Malignant lymphoma involving spinal cord (photograph)
Ford (1952)	Medulloblastoma (no description)

The last two of Larson, Robson, and Reberger's (1953) five cases are excluded because of the absence of any evidence of meningeal spread and the paucity of the data. Cases 2 and 3 are also not very convincing, but have been included; there is no mention of the state of the meninges post mortem.

Viklicky (1952, Case 2) has described a case of medulloblastoma with abnormal cells in the C.S.F.,

TABLE II

SECONDARY TUMOURS OF THE CENTRAL NERVOUS SYSTEM

Site of Primary Growth	Author	Type of Growth and Remarks
Stomach	Widal and Abrami (1908)	Carcinoma (drawings)
	Stadelmann (1908)	Carcinoma
	Schwarz and Bertels (1911)	Probably carcinoma of stomach. Necropsy only allowed on brain
	de Fortunet and Cade(1916)	Carcinoma
	Pette (1922, 2nd case)	,,
	Wüllenweber (1923)	,,
	Penecke (1927)	,,
	Schroeder *et al.* (1945, 1st case)	,, (photographs)
	McCormack *et al.* (1953, Case 2)	,, (photograph)
	McCormack *et al.* (1953, Case 6)	,, ,,
	Chesterman and Semple (1953)	,, (photographs)
	Meissner (1953)	,, (photograph)
Lung ..	Carnot and Baufle (1911)	" Fuso-cellular sarcoma "
	Peter (1924)	Carcinoma (photographs)
	Bertha (1935, 1st case)	Pleomorphic carcinoma (photograph)
	Ginzburg (1941)	Adenocarcinoma (drawings)
	Schroeder *et al.* (1945, 3rd case)	Carcinoma. Fluid obtained by ventricular puncture (photograph)
	Dostál (1949)	Anaplastic carcinoma (photograph)
	Viklicky (1952, Case 1)	Adenocarcinoma (photographs)
	McCormack *et al.* (1953, Case 4)	Adenocarcinoma (photograph)
	McCormack *et al.* (1953, Case 5)	Adenocarcinoma (photograph)
	Larson *et al.* (1953, Case 1)	Carcinoma
	,, ,, ,, ,, 3)	Oat-cell carcinoma (photograph)
Breast	Humbert and Alexieff(1913)	Carcinoma (drawing)
	Lemierre and Boltanski (1929)	,, (drawings)
	Piaggio Blanco and Paseyro (1945)	,, (photographs)
	Pinto (1946, Case 1)	,, ,,
	Nayrac *et al.* (1950)	,,
	McCormack *et al.* (1953, Case 7)	,, (photograph)
Kidney	Fischer (1922, Part I, Case 5)	Hypernephroma
	Fischer(1922 Part II,Case 4)	,,
	Bertha (1935, 2nd case)	Adenocarcinoma(photograph)
Pancreas	McCormack *et al.* (1953, Case 1)	Carcinoma (photograph)
	McCormack *et al.* (1953, Case 3)	,, ,,
	Larson *et al.* (1953, Case 2)	Adenocarcinoma (photograph)
Eye ..	Panton (1922)	Round-celled sarcoma
Skin ..	Boyd (1925)	Melanoma
	McCormack *et al.* (1953, Case 9)	,, (photograph)
Omentum	Facchini (1908)	" Endothelioma "
Thymus	Danisch and Nedelmann (1928)	Malignant thymoma
Thyroid	Fischer (1922, Part I, Case 1)	Carcinoma, unproved (drawings)
Primary growth unknown	Meyer (1911 and 1922)	? Adenocarcinoma of uterus
	Pette (1922, 1st case)	Adenocarcinoma, ? from parotid tumour
	Fischer (1922, Part I, Case 2)	Unproved; probably carcinoma of bowel
	Forster (1930, 12th case)	Anaplastic carcinoma (photograph)
	Pinto (1946, Case 2)	No histology (photographs)
	McCormack *et al.* (1953, Case 8)	Carcinoma (photograph)

and post-mortem proof of meningeal dissemination. Although the photographs are suggestive of tumour cells Viklicky does not accept them as such, and this case is therefore not included.

In Kono's (1924) case of choroid plexus tumour, malignant cells were only found in C.S.F. removed by lumbar puncture after death. He published no illustrations.

Fischer's (1922) nine cases have all been admitted, but the evidence in several of them is onlv slender.

Material and Methods

In the present series of seven cases, in which tumour cells were found in the C.S.F., all specimens of fluid were examined routinely in the Clinical Pathology Laboratory, Radcliffe Infirmary. During the period when they were seen, films were only made if unusual cells were seen in the counting-chamber, or if the examination for malignant cells was requested. It is likely that other cases were missed, owing to our failure to make stained films in all cases of pleocytosis.

All the cases in this series have been studied by means of air-dried films stained by Romanowsky stains. Although such films are not always easy to make, their interpretation is relatively straightforward to anybody accustomed to Romanowsky films of blood and marrow. The Papanicolaou method as applied to C.S.F. has not been found very satisfactory, and wet-chamber methods, such as that recently advocated by McCormack, Hazard, Gardner, and Klotz (1953), have the serious drawback of impermanence.

The method used is as follows:

As much C.S.F. as can be spared for the examination is centrifuged in a conical tube until the cells are deposited; the supernatant fluid is poured off and can be used for other estimations. After pouring off the supernatant the tube is inverted so that no C.S.F. can run back on to the cells. The cellular deposit is then removed with a platinum loop. Usually a pin-head-sized portion can be made to adhere to the tip of the loop, and this is deftly spread out on a clean slide. If the cells are so scanty that there is no deposit removable in this way, this method cannot be used. The smear seldom covers more than 1 sq. cm. Essential points are: (1) That the smear should dry instantaneously; if it takes a minute to dry, owing to excess of fluid, it may as well be discarded. (2) That no part of the smear should be scratched with the loop after it is dry.

The air-dried films are then fixed in methyl alcohol for three minutes and stained with Leishman's stain or with May–Grünwald–Giemsa in exactly the same way as blood or marrow films. May–Grünwald and Giemsa as described on page 402 of Dyke's *Recent*

Advances in Clinical Pathology (2nd ed., Churchill, 1951) has been used in this investigation. Where red cells are numerous the method of choice is to centrifuge, remove the supernatant fluid, add a little fresh cell-free compatible serum (less than a drop), and make films exactly as for blood. The films from Case 7 in this series were made by Dr. J. B. Howie by this method. Films made by spreading without serum replacement are apt to be slow-drying and this impairs the subsequent staining.

In cases where sufficient cellular deposit can be obtained these methods give excellent results. A satisfactory way of making stained films from samples with very low cell counts remains to be devised. Some success has been obtained using a fine squirrel-hair paint-brush to transfer the deposit to the slide.

Case Reports

Case 1 (121640).—A woman aged 55 was admitted to hospital on March 20, 1951, with a complaint of attacks of "beating" in the head for three months. The attacks had been increasing in severity, with giddiness and a sensation of pressure behind the eyes. When first admitted she had no neurological signs except for weakness of the right leg. The C.S.F. pressure was 160 mm. Hg, the protein 45 mg.%, and the cells 19 c.mm. with 12 lymphocytes and 7 polymorphs. A diagnosis of migraine was made. She was admitted again on April 11, 1951, with partial blindness and retention of urine. At this time there was gross diminution in the visual fields, loss of reaction of the left pupil, bilateral external rectus palsy, slight left facial weakness and a blood pressure of 155/120 mm. Hg. The margins of the optic disc were blurred. Lumbar puncture produced clear fluid at a pressure of 100 mm. Hg, protein 80 mg.%, cells 35/c.mm. (33 lymphocytes, 2 polymorphs).

Rectal examination revealed a firm rounded mass in the pouch of Douglas, and a provisional diagnosis was made of ovarian carcinoma with intracranial metastases.

Lumbar puncture performed on April 17 produced clear fluid with a very light deposit of cells after centrifuging. These were mostly red cells, with lymphocytes, macrophages and some atypical cells reported as highly suggestive of malignancy.

Atypical Cells.—These cells were large (up to 24 μ diameter), with large round nuclei (12–16 μ diameter), in some cases showing an ill-defined nucleolus, and moderately profuse foamy or cloudy cytoplasm. They did not adhere in clumps (Fig. 1). No mitotic figures were seen.

A necropsy performed on May 7 (P.M. 253/51) showed a primary adenocarcinoma of the recto-sigmoid junction, with multiple secondaries. There was diffuse metastatic carcinomatosis of the meninges over the brain and spinal cord, histologically mostly undifferentiated in type but in places showing an adenocarcinomatous pattern.

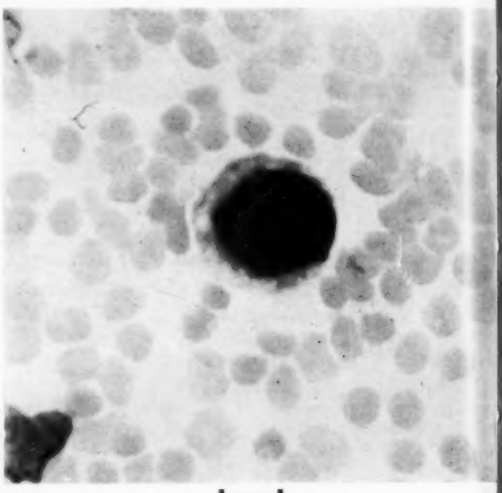


FIG. 1.—Case 1 (carcinoma of the colon): a large carcinoma cell lying among red cells. It is easily identifiable by the size of its nucleus and the high nucleo-cytoplasmic ratio.

Case 2 (141793).—A woman aged 58 was admitted on May 22, 1951, with seven weeks' history of mid-thoracic girdle pains, and one week's history of weakness of the legs and difficulty in passing urine. She was found to have a sensory level at T 4 and complete flaccid paralysis below this level. There was no evidence of a primary growth outside the spine, and radiographs showed a paravertebral shadow in the mid-thoracic region. Lumbar puncture produced clear fluid with a protein content of 600 mg.% and no cells. Films of centrifuged deposit were not made. Myelography revealed a spinal tumour, and laminectomy was performed on May 24. A biopsy was taken of an extrathecal tumour, and the sections showed plasma cell myeloma. She died on May 26.

Cerebrospinal fluid obtained at the time of laminectomy was centrifuged and films were examined.

Atypical Cells.—The films showed numerous large abnormal cells, closely resembling a type of "myeloma cell" seen in the bone marrow (Fig. 2). In most cases they lay separately from one another, but sometimes in small aggregates. The whole cell in mononuclear examples measured 14–22 μ across, but multinucleate cells sometimes reached 60 μ in diameter. The nucleus usually had a diameter of 10 to 14 μ, but was sometimes as large as 22 μ across; it lay eccentrically in most cases, and had an even but rather coarse chromatin structure. There were one or several irregular large nucleoli, sometimes measuring even up to 8 μ across. The cytoplasm was stained deep blue, except for a juxta-nuclear area of pallor, and was of ground-glass texture. There were occasionally a few punched-out

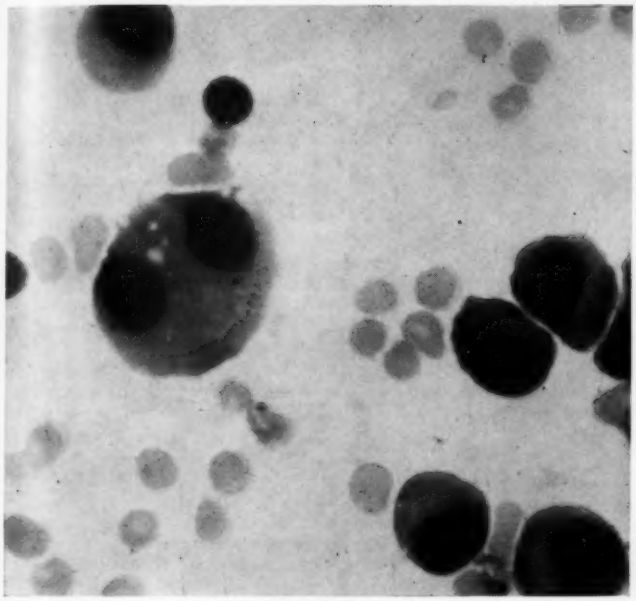


FIG. 2.—Case 2 (plasmacytoma) : characteristic myeloma cells with red cells and a few lymphocytes.

vacuoles. Most cells were mononuclear, but cells with 2 to 4 nuclei were present and a few mitotic figures were seen.

Associated cells were erythrocytes and a few lymphocytes.

At necropsy on May 28 (P.M. 294/51) an extradural tumour was found arising in the ninth thoracic vertebra and extending extradurally from T 1 down to T 10. Histologically this was a plasma-cell myeloma. The meninges showed only slight macroscopical granularity over the spinal cord, and microscopically scattered isolated myeloma cells were present in the subarachnoid space.

Case 3 (142365).—A boy aged 14 had about three months' history of pain in the neck and failure of vision in the right eye, with headaches and some vomiting. Radiological studies showed a defect in the anterior fossa, which was explored on June 7, 1951. A diffuse arachnoid cyst was found, the nature of which was not determined. He returned a month later with further failure of vision, and lumbar puncture on July 31 produced turbid yellow fluid under less than atmospheric pressure, with a protein content of 580 mg.%. There were 16,000 red cells and 34 white cells per c.mm.

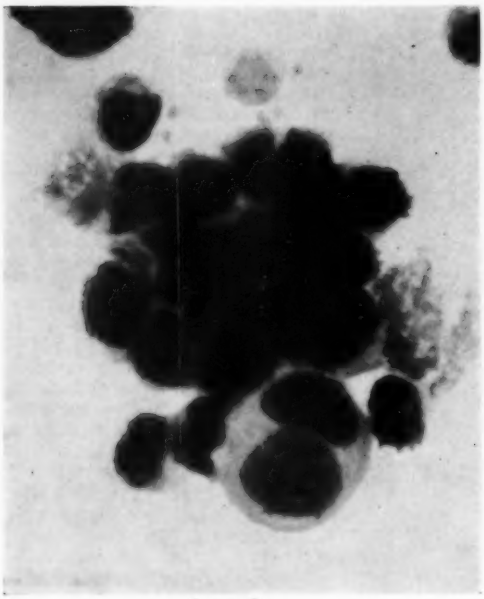
Atypical Cells.—Almost all the white cells were of the same type. They measured 10–18 μ across ; the nuclei were round or irregular, almost filling the cell, with a fine chromatin structure, and with 1 to 3 nucleoli each measuring about 3 μ across. The cytoplasm was in most cases rather scanty, moderately basophilic and almost clear. In some cases there were a few vacuoles. These cells occurred in clumps of dozens or even hundreds together (Fig. 3). No mitotic figures were observed.

Similar cells were seen in subsequent specimens of C.S.F. up till the time of transfer to the Royal Berks Hospital, Reading, on August 13. *X*-ray treatment was given for a supposed cerebral tumour. The boy died on April 27, 1952, and a necropsy (Dr. J. Mills) revealed bilateral soft paravertebral tumours extending downwards from the tenth thoracic vertebra and infiltrating the vertebral bodies. The brain and spinal cord were sent here for examination (S.H. 3218/52). The arachnoid cysts did not appear to be neoplastic, but there was massive infiltration of the spinal subarachnoid space with tissue identical with that of the paravertebral masses. Histologically the growth was a poorly differentiated connective tissue tumour, probably of a reticulosarcomatous nature.

Case 4 (159933).—A girl aged 4 was admitted to another hospital on May 12, 1952, with headache, vomiting, and left-sided weakness of four weeks' duration. Left-sided weakness was confirmed, and a lumbar puncture produced fluid under 90 mm. Hg pressure, with a protein of 30 mg.% and 11 cells per c.mm. She was regarded as a case of hemichorea. She was admitted again with headache and vomiting on June 19 and the C.S.F. was now under a pressure of 270 mm. Hg, bloodstained, with a protein of 180 mg.%, chlorides 685 mg.%, no sugar, and 55 white cells per c.mm., reported as lymphocytes. No acid-fast bacilli were found.

On transfer here on June 21 she was found to have left-sided weakness and incoordination so that she was unable to walk. Lumbar puncture on June 24 produced turbid yellow fluid under 300 mm. Hg pressure. The protein was 660 mg.%, chlorides 660 mg.%, sugar absent. There were 2,000 red cells and 500 white cells per c.mm.; almost all the latter were reported as tumour cells. Ventriculography the same afternoon showed a large tumour below the right lateral ventricle ; smears of material aspirated from the tumour were reported by Dr. D. Bosanquet as follows :

"The smear shows much necrotic debris, scattered through which are tumour cells lying singly or in small clumps. These cells have little cytoplasm and



10μ

FIG. 3.—Case 3 (reticulosarcoma): collections of tumour cells with very scanty cytoplasm. A single large binucleate cell has profuse cytoplasm and prominent enlarged nucleoli.

round vesicular nuclei with a well-marked nucleolus and nuclear membrane. Mitoses are present in moderate numbers. The appearance is unusual and the cells do not look like those from a medulloblastoma or any of the commoner gliomas; it is possible that the primary tumour is outside the central nervous system, but no diagnosis can be made other than that it is certainly a malignant tumour."

A further two specimens of C.S.F. both showed the same type of cells as had been seen before. The child died on June 28, and permission for necropsy was refused.

Atypical Cells.—The cells either lay separately from one another or aggregated into clumps of up to 30 together (Fig. 4). Each cell measured from 12 to 22 μ across. The nucleus was polygonal or irregular in shape, 10 to 20 μ in diameter, with a fine chromatin structure and one or more well-marked nucleoli staining pale blue and sometimes measuring as much as 4 μ across. The cytoplasm was scanty, staining deep blue, and sometimes showing a large vacuole near the cell centre and a few small punched-out vacuoles probably due to fat. Mitoses were not observed.

Case 5 (162892).—A boy aged 2½ was admitted to another hospital on August 30, 1952, with two or three weeks' history of impaired appetite and a week's history of vomiting and drowsiness. He became unconscious on the day of admission. Clinically he showed mengingitic signs, and lumbar puncture produced fluid under more than 300 mm. Hg pressure. The protein was 89 mg.%, chlorides 756 mg.%, sugar content normal. There were 114 cells per c.mm., six being polymorphs and 101 interpreted as lymphocytes. No acid-fast bacilli were found. He was transferred to the Radcliffe Infirmary on August 31, as a possible case of tuberculous meningitis, and C.S.F. obtained by lumbar puncture here contained 110 mg.% of protein, 660 mg.% of chlorides, normal sugar, and 1,300 cells per c.mm. Many of these cells occurred in clumps of 10 to 20; they were at first thought to be lymphocytes, but stained films showed them to be tumour cells.

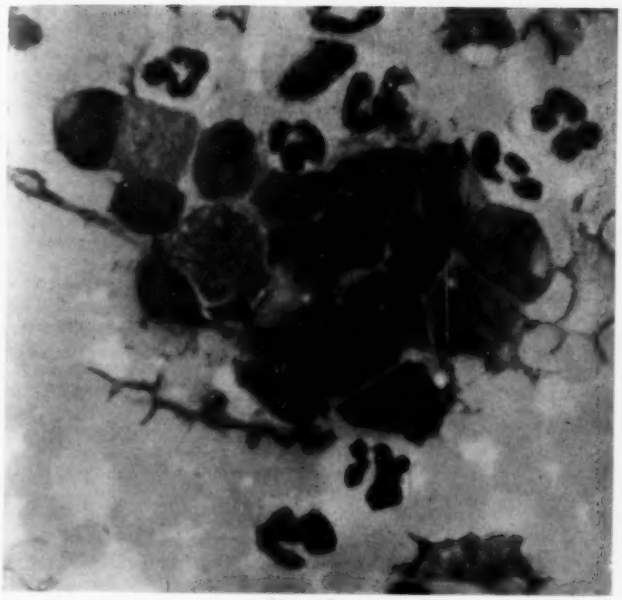
A ventriculogram done the same day showed a deformity of the fourth ventricle, and on September 1 exploration and decompression were performed. The condition was found to be a cerebral tumour with meningeal spread; a biopsy showed a medulloblastoma.

A post-operative specimen of C.S.F., besides abundant red cells, contained 1,800 white cells per c.mm., almost all tumour cells (Fig. 5).

Atypical Cells.—The cells referred to as tumour cells were not very large, measuring only 9 to 15 μ across. The nucleus almost filled the cell. Its

10μ

FIG. 4.—Case 4 (primary tumour not identified): a collection of very pleomorphic tumour cells. There is a strikingly high nucleocytoplasmic ratio, and several cells appear to have secretory vacuoles.



10μ

FIG. 5.—Case 5 (medulloblastoma): a clump of medulloblastoma cells among closely packed red cells and neutrophils. The tumour cells are larger than lymphocytes and have very scanty cytoplasm; their nuclear chromatin is fine and nucleoli are visible. They could easily be confused with monocytes or large lymphoid cells, but their occurrence in clumps and their fine chromatin pattern are distinguishing features.

chromatin structure was fine, and each nucleus contained one or more relatively large nucleoli, sometimes as much as 4 μ in diameter. No mitoses were seen. The cytoplasm was scanty and moderately basophilic. Both nucleus and cytoplasm often showed a few very small clear vacuoles.

These cells occurred in clumps, often of dozens together, and owing to their scanty cytoplasm the nuclei were closely applied to one another, producing a mosaic-like effect.

The child died, and a necropsy (P.M. 452/52) showed massive subarachnoid spread of a medulloblastoma arising in the cerebellum.

Case 6 (166531).—A woman aged 52 was admitted on November 14, 1952, with a history of lumbar pain for several days in August, thought to be due to a prolapsed disc, and subsequent abdominal pain and vomiting. A few days before admission she became disorientated and suffered from frontal headache. The principal findings on examination were slight left facial weakness, right foot drop, and a little pain on right straight-leg-raising. She was mentally confused and uncooperative.

Lumbar puncture on November 15 produced yellow "syrupy" fluid under 180 mm. Hg pressure. The protein content was above 1,000 mg. per 100 ml., and clot was present after standing; there were 300 red cells and 15 white cells per c.mm., the latter consisting of seven neutrophils and eight lymphocytes. Another lumbar puncture on November 18 showed a similar finding.

A ventriculogram showed no space-occupying lesion.

On November 25 at lumbar puncture a lightly bloodstained yellow fluid was obtained containing over 1,000 mg. of protein per 100 ml. It clotted spontaneously. There were 2,500 red cells and 22 white cells per c.mm.; of the latter, 10 were neutrophils, 8 lymphocytes, and 4 "atypical" cells. For the first time, stained films were made and the atypical cells were reported as probably malignant. They were found again in subsequent specimens (Fig. 6).

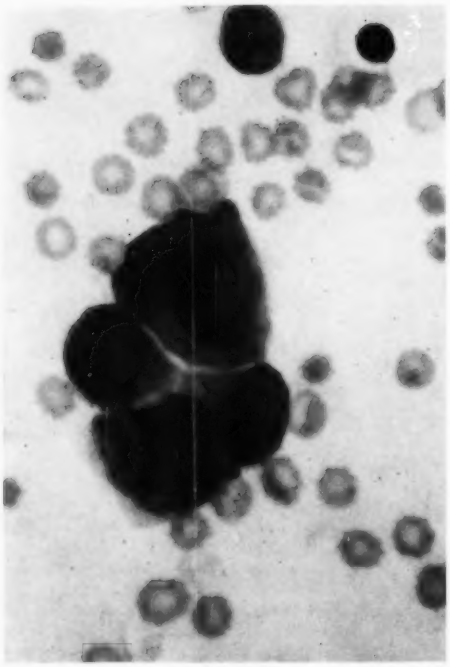
Atypical Cells.—The cells were isolated from one another and measured 12–22 μ across. The nucleus was generally single, but in some cells 2 to 4 nuclei were present. When single, the nucleus usually lay to one side of the cell, and was round or irregular, measuring anything from 10 to 20 μ in diameter. The chromatin pattern was fine, and there were up to five ill-defined nucleoli of normal size. The cytoplasm was scanty, staining greyish blue, with a ground-glass appearance. Sometimes there was a vacuole next to the nucleus, but this was not stained by the periodic-acid-Schiff method following salivary digestion, and was therefore probably not mucus-secreting. None of these cells were seen in mitosis.

The patient became comatose and died on December 2.

At necropsy (P.M. 606/52) the whole cord and cauda equina and the subarachnoid cisterns of the brain were found to be enveloped in a gelatinous exudate, and there was a tumour in the region of the pineal gland and slight enlargement of the left thalamus. No primary tumour was found outside the central nervous system.

Histologically the tumour proved to be a glioma spreading as a diffuse astrocytoma through the pulvinar on one side and showing anaplastic change to become a glioblastoma multiforme near the posterior surface, whence it involved the leptomeninges.

Case 7 (171953).—A woman aged 57 was first admitted in March, 1953, with a year's history of lassitude and vomiting, and about six weeks' history of headaches, failure of vision, and loss of weight. She had also had several fits. She was found to be hypertensive and to have tenderness over the sub-



10μ

FIG. 6.—Case 6 (glioblastoma multiforme): a group of four tumour cells. They are pleomorphic, and two of the nuclei are well beyond the normal limit of size for macrophages. The nuclei have a fine "primitive" chromatin pattern, and the cytoplasm is scanty.

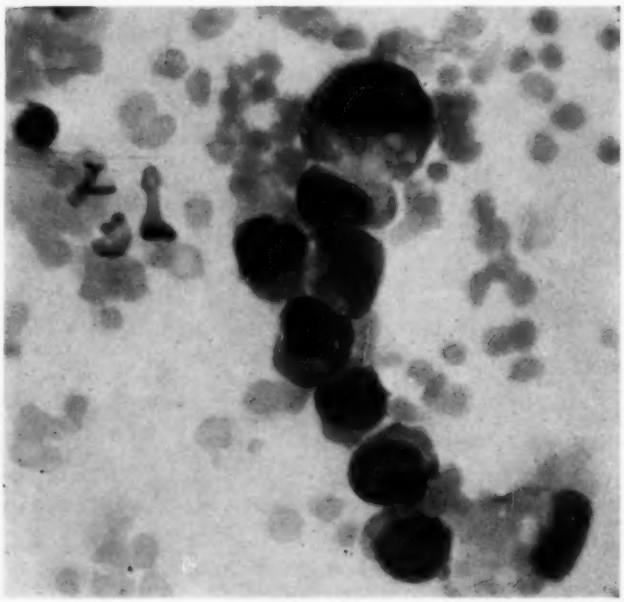
occipital and temporal regions of the scalp. She was blind, with retinal pallor, and had some weakness of the right leg and face. Lumbar C.S.F. was at 170 mm. pressure, with protein 50 mg.% and 11 cells (7 polymorphs and 4 lymphocytes) per c.mm.

A radiograph of the skull showed erosion of the dorsum sellae. An air encephalogram, ventriculogram, right angiogram, and temporal artery biopsy were uninformative. On April 5 she began to lose C.S.F. from the right post-parietal burr-hole, and when the wound was excised on April 9 a brain fungus was seen. From April 15 she had repeated epileptic fits.

On April 27 a specimen of C.S.F. was reported to contain numerous abnormal cells which could only be regarded as malignant. Because these cells were not very large, the opinion was given that the appearances were more in favour of a tumour arising in the central nervous system rather than of a metastatic carcinoma. The following is a description of the film of centrifuged deposit:

Red cells were abundant. White cells were also numerous, and a differential count gave tumour cells, 43%, lymphocytes, 20%, neutrophils, 23%, macrophages, 14%.

Atypical Cells.—The tumour cells (Fig. 7) were pleomorphic and were separate or occasionally in small groups; the nucleus was 12–18 μ in diameter, irregularly round or oval, with a chromatin pattern similar to that of a macrophage but more deeply staining, and in some examples an ill-defined



10μ

FIG. 7.—Case 7 (adenocarcinoma of the lung): a row of tumour cells showing variations in size and, in some cases, a high nucleocytoplasmic ratio. A lymphocyte nucleus is shown above and to the left.

nucleolus was visible. The nucleo-cytoplasmic ratio was often high, but in some cells the cytoplasm was fairly profuse; the total diameter of the cell was from 15 to 24 μ, usually 16 to 18 μ. The cytoplasm was moderately basophilic, cloudy, sometimes containing fat vacuoles. Here and there a cell showed an ill-defined vacuole at the cell centre which appeared to deform the nucleus. The periodic-acid-Schiff stain on ptyalin-digested slides did not demonstrate any mucus in these vacuoles. A few cells in mitosis were present. The "macrophages" were large and in some instances perhaps represented degenerating tumour cells.

No other evidence of a neoplastic process was obtained during life.

The patient was transferred to Stoke Mandeville Hospital on May 7 and died two months later. At necropsy (Dr. H. J. Harris) she was found to have a bronchial carcinoma. The meninges showed only slight macroscopic thickening, but sections showed very extensive infiltration of the subarachnoid space with adenocarcinoma.

Discussion

Malignant cells cannot be distinguished by any single morphological feature. The diagnosis of malignancy from isolated cells can be either easy or very difficult according to the type of growth and the cellular environment. In the case of C.S.F. there is not a very large variety of cell types from which tumour cells have to be distinguished, and in well-stained films the diagnosis is sometimes obvious. The following are the principal criteria on which the diagnosis is based: (1) The cells are foreign to their environment: they differ from any type of cell occurring in the C.S.F. in other conditions. (2) The cells morphologically resemble those seen in particular varieties of tumour: for example, in Case 2 they were recognizably like "myeloma cells" of the bone marrow. (3) The cells may show some of the features characteristic of tumour cells in general: of these the most noteworthy are large size, especially of the nucleus; high nucleo-cytoplasmic ratio; reduplication of nuclei; enlargement of nucleolus; great variation in size and shape of otherwise similar cells; and frequent mitoses.

None of the above features is present in every case, and none by itself is of particular significance. The cases described here and the descriptions in the literature are not numerous enough to allow many generalizations to be made about even the commonest types of malignant cells seen in C.S.F. It seems that metastatic carcinoma cells are usually exceptionally large, with absolutely and relatively large nuclei, but adenocarcinoma cells show much less tendency to form morular or acinar structures than they do in pleural or peritoneal effusions. The small anaplastic cells of medulloblastoma are distinguished by a "primitive" chromatin pattern, high nucleo-cytoplasmic ratio, and occurrence in aggregates. Mitotic figures were seen in only Cases 2 and 7, and are not to be expected in most types of tumour cell; they also occur in non-malignant fluids, particularly among plasma cells, and their presence is therefore of little diagnostic help.

In all the seven cases of the series lumbar puncture had been previously performed, sometimes repeatedly, and yet malignant cells had not been discovered because they were not looked for. In Cases 1, 2, 4, and 5 the diagnosis was made by other means either before or at the time of discovery of malignant cells. In Cases 3, 6, and 7 the finding of malignant cells in the C.S.F. was the only firm evidence of a malignant tumour to be obtained before death. In Case 7 the diagnosis of neoplastic infiltration of the meninges was thought unlikely on clinical grounds even after the cytological findings had been reported. No evidence was forthcoming until necropsy was performed. Case 5 is particularly instructive in that the symptoms and signs mimicked those of tuberculous meningitis, and the initial finding of considerable numbers of "lymphocytes" supported this diagnosis. Subsequent examination of a stained film showed that the cells bore very little morphological resemblance to lymphocytes.

It must be emphasized that mistakes can only be avoided if the examiner is thoroughly familiar with the cells of non-malignant conditions studied by the same technique, and a certain degree of skill in the making of the films is also essential.

Once this experience has been acquired, the examination of stained films for tumour cells can be a valuable, simple, and rapid diagnostic procedure, and is to be recommended in all cases of cerebrospinal pleocytosis where the cause is not already known.

Summary

Seven cases are described in which tumour cells were recognized in the cerebrospinal fluid during life. In six cases there was post-mortem proof of meningeal dissemination of malignant growth, and in the seventh necropsy was refused.

Of the six complete cases, two had primary cerebral tumours (a glioblastoma multiforme and a medulloblastoma), two had adenocarcinomata, one had a reticulosarcoma, and one had a myeloma as the primary growth.

Sixty-six other cases have been found in the literature.

A method for making Romanowsky-stained films of C.S.F. deposits is described, and the preparation of such films is recommended in cases of pleocytosis of doubtful cause.

I wish to thank Dr. A. H. T. Robb-Smith and Dr. P. D. Daniel for their advice and encouragement; Dr. Dawn Bosanquet for most of the post-mortem examinations and histology, and for much valuable help; Dr. J. Mills and Dr. H. J. Harris for the post-mortem reports on Cases 3 and 7 respectively; Mr. Joe Pennybacker for permission to publish the case reports; and Mr. K. E. White, who in several cases first detected tumour cells in the counting chamber.

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A CASE OF ANOREXIA NERVOSA WITH NECROPSY FINDINGS AND A DISCUSSION OF SECONDARY HYPOPITUITARISM

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Anorexia nervosa is seldom fatal, so there are few necropsy reports in the literature. The case reported here is of interest partly because it came to necropsy, and partly because the necropsy findings have a bearing on the question, much discussed in the literature, whether hypopituitarism may result from the inanition of anorexia nervosa.

It may seem unfortunate that so long a time has elapsed between the patient's death (1938) and the publication of this report. In those days hormonal assay was much less advanced than now, and the patient's unexpected death prevented much being done. Further, the war caused the matter to be set aside, not to be taken up again for more than a decade. However, so much of relevance has been learnt since those days that perhaps the case is even more interesting now than it was then.

Case Report

The patient, K. L., a woman of 26, was admitted to Willesden General Hospital, under Dr. S. Leonard Simpson, on March 4, 1938, and died on March 8. She had previously been admitted to the London Hospital in 1935. The necropsy was performed by Dr. G. E. Harre.

In 1931, when K. L. was 19, she was discharged from work, as her employers were cutting down staff. She was very disappointed. Her periods suddenly stopped, and she began to lose weight. Her weight before her illness had been 120 lb. In 1935, when she was 23, she weighed 77 lb., and, in 1938, just before her death, 70 lb. Her own reports about her appetite are inconsistent.

She was very active even to the last; people stared to see someone so ill getting around so briskly. There had been partial loss of pubic and axillary hair.

She had chorea at the age of 7, and acute rheumatism at 18. She left school at 14 and started work at 15. She was happy at home and at work, but was not a good mixer. She led a sheltered life. At 18 she was good looking, rather plump, fond of music, and anxious to go to the Royal Academy of Dramatic Art, but her parents were too poor.

On her first admission, to the London Hospital in 1935, examination showed an extremely wasted girl. There was no abnormal pigmentation. The pulse was 60, blood pressure 90/70–106/70 mm. Hg. The heart, lungs, and central nervous system were normal. On pelvic examination the ovaries were not felt, and the uterus was reduced to the thickness of an index finger.

The blood count was within normal limits. The plasma protein was reduced to 5 g./100 ml., serum calcium was 10.5 mg./100 ml. Two glucose tolerance curves were done. The first showed a fasting level of 80 mg./100 ml., followed by 115 mg. at 30 minutes and 115 mg. again at 60 minutes. The second curve began at 77 mg./100 ml., fasting level, followed by 220, 190, 220, and 180 mg. at half-hour intervals. There was no glycosuria. Radiographs of the chest and long bones were normal. The B.M.R. on two occasions was 67% and 85% of normal.

On her second admission to hospital, to Willesden General Hospital in 1938, she was described by the house-surgeon as "skin and bones." Her skin was dry, scaly, and pallid, and puffy under the eyes and over the ankles. The scalp hair was brown, coarse, and dry; the eyebrows were well defined; pubic and axillary hair was present but somewhat scanty. Heart sounds were normal; the pulse was 60, the blood pressure 104/62 mm. Hg.

She was bright and alert. There was no indication on March 7, after three days in hospital, of any immediate danger to life. However, at 6.30 a.m. the next morning the nurse could not rouse her; at 8 a.m. it was realized that she was in coma. She died soon after. Clinically it seemed that she was in hypoglycaemic coma. (A sample of blood taken at necropsy contained no sugar.)

Diagnosis.—Clinically, there seems no doubt that the diagnosis of anorexia nervosa was accurate, and the case rather typical.

In spite of considerable emaciation, she manifested great activity and restlessness, in contrast to the apathy and inertia of Simmonds's disease. The growth of fine down on the face was in keeping with the diagnosis of anorexia nervosa; in this and other states of inanition, including famine, such downy hair may

grow over the whole body. It is not met with in Simmonds's disease.

Complete or almost complete loss of pubic and axillary hair is usual in Simmonds's disease and invariable if the pituitary insufficiency is severe. In contrast, persistence of pubic and axillary hair is characteristic of anorexia nervosa, even of severe degree.

Post-mortem Findings

External Appearances.—The body was that of an extremely emaciated young woman. The axillary hair was sparse, as was that over the mons pubis, but there was more on the vulva. The scalp hair was thin. There was some soft hair over the angle of the jaw.

Cardiovascular System.—The myocardium was brownish. The right ventricle was 0.3 cm. thick, the left ventricle 0.9 cm. The mitral cusps were pale, and showed bulbous thickenings. The heart as a whole was very small.

Respiratory System.—The lungs were somewhat brown, and dry on cutting ; there was fine emphysema.

Alimentary System.—The lingual and pharyngeal tonsils were very small. The stomach contained thick, bile-stained chyme and much mucus. The gut generally was contracted. The liver was small (453 g.), its colour markedly brown, its pattern very small. The gall bladder and bile ducts were normal. The pancreas was small (41.5 g.) and of a deep yellowish-brown.

Haemopoietic and Endocrine System.—The lymph glands generally were small, save for some calcified mesenteric glands. The spleen weighed only 38.7 g.; it was moderately firm, with a finely wrinkled capsule. On section the fibrous tissue was conspicuous, the Malpighian bodies extremely small. The thymus had apparently atrophied completely. The thyroid was small and pale. The suprarenals, weighing together 9.1 g., were firm and reddish-yellow ; on section, lipoid and pigment zones could be seen, though the latter was thin. The medulla was easily discerned. The pituitary appeared normal ; it weighed 0.7 g. and measured $1.25 \times 0.9 \times 0.6$ cm.

Urinary System.—The kidneys were small and firm ; the right kidney weighed 71 g., the left 63.5 g. The capsules stripped readily, and the pattern was good.

Female Genitalia.—The uterus was small, being 4.7 cm. long, of which the cervix made up 2.2 cm.; the endometrium was smooth. The ovaries were small and very firm, with cut surfaces almost devoid of pattern.

Nervous System.—The brain (1,335 g.) appeared normal externally.

Chemical Analysis.—A specimen of blood was obtained about three and a half hours after death and taken immediately for estimation of sugar. No sugar was found. (Three control tests were done on other cases, the samples being taken six, nine, and 17 hours after death ; the blood sugar was 36, 122, and 33 mg. per 100 ml. respectively). The non-protein nitrogen was 64 mg. per 100 ml., the total protein 5.16 mg., and the blood chloride 383 mg. per 100 ml.

Histological Findings

Pituitary.—It was obvious from haematoxylin and eosin sections that the gland was abnormal. Nearly all cells showed faint eosinophilia ; lightly staining granules were visible with the 1/12 in. objective. There was no scarring.

The gland was treated on the lines indicated by Crooke and Russell (1935). In view of the histological abnormality of this pituitary every section was stained in exact parallel with a normal control (Figs. 1 and 2).

The glandular cells fell into four groups. One group was of cells containing fuchsinophil

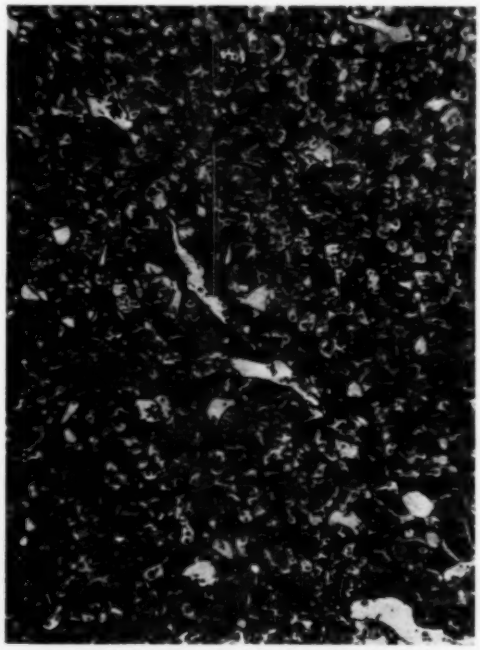

FIG. 1.—A typical low-power field of the pituitary of this case, stained by acid fuchsin-aniline blue-orange G. × 95.

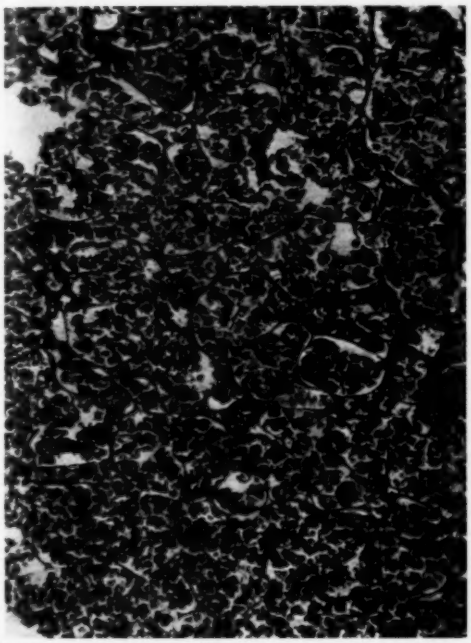

FIG. 2.—A typical low-power field of the normal control stained in parallel with that shown in Fig. 1. × 95.

granules. The granules were in most cases very scanty and in only a few cells did they fill the cytoplasm in the way seen in normal acidophil cells. A second group was of cells having cytoplasmic granules staining with orange G. Here again the granules were generally scanty. A third group was of cells containing granules staining with aniline blue, as do the basophil cells in the normal pituitary. Most of these were filled with granules. The fourth group was of cells containing no granules, or occasionally granules not staining at all. Most of these cells had scanty cytoplasm ; in many cases they appeared as " groups of nuclei embedded in a common cytoplasm," to quote the description given to some of the normal chromophobe cells by Bailey (1932).

The percentages of the various cell types were:

Fuchsinophil (acidophil) cells	15.3
(apparently normal acidophil cells included in above)	(1.26)
Orange G cells	42.5
Basophil cells	4.64
Chromophobe cells	37.4
	99.84

Sections were also treated by the periodic-acid-Schiff (P.A.S.) technique described by Pearse (1949) for the localization of gonadotrophic hormone. A count was done of P.A.S.-positive cells in a selected strip right across one of the sections, and also of basophil cells in the corresponding part of the next section in the series, stained by the acid fuchsin-aniline blue-orange G method. It was found that the basophil cells were P.A.S.-positive but that many P.A.S.-positive cells were not shown up as basophil cells by the latter method. This conforms with Pearse's findings. (For 135 basophil cells in the one section there were 459 P.A.S.-positive cells in the other.)

Adrenals.—The cortex was generally narrow. In one section the thickness was 0.6 mm. (mean of measurements at 15 places), in another 0.75 mm. (mean of 24 measurements). The zona glomerulosa and zona reticularis in particular were very narrow, their component cells often atrophic, with pyknotic nuclei. In some places these zones had almost or altogether disappeared ; where this was the case the zona fasciculata was especially thin, though this zone was everywhere the least atrophic of the three. The capsule of the gland was generally thicker than normal, and most thickened where the cortex was most thinned. Collagen fibres were present in excess among the cells of the zona reticularis, and pigment in these cells was scanty. The medulla was normal.

Ovaries.—The cortex was very thin. There were a few old corpora alba. A small number of primordial follicles were found ; no follicle showed the slightest degree of maturation.

Myometrium.—There was great atrophy of the muscle fibres.

Liver.—The lobules and the cells were small. A large amount of iron-containing pigment was present, some in the liver cells, some in the Kuppfer cells. There was no cirrhosis.

Spleen.—The most striking change was the great amount of iron-containing pigment, some of which was loose in the connective tissue of the sinusoidal walls, some in histiocyte-like cells scattered through the pulp. The sinusoids were moderately dilated and were lined by low epithelium-like cells ; there was increased connective tissue in their walls. The Malpighian bodies were very small.

Pancreas.—The islets appeared small and scanty. Most of the islet cells were small, but otherwise appeared normal.

The exocrine cells appeared normal apart from being rather small.

Heart.—The general picture was that of brown atrophy.

Stomach.—The mucosa was thin but histologically normal. The lymphoid follicles were very scanty and small.

Tonsils and Lymph Glands.—The amount of lymphoid tissue was remarkably small ; only a few of the very small follicles had germ centres, and these too were very small. The sinusoids were dilated.

Kidneys.—There was much congestion. The glomeruli and tubular epithelial cells appeared smaller than normal. The kidneys were otherwise normal.

Discussion

Conflict of Views.—In the literature, much discussion is devoted to the relationship, if there is one, between anorexia nervosa and Simmonds's disease. Many conflicting statements are made, many conflicting opinions expressed. Some say "there is no reason to believe that pituitary deficiency exists in anorexia nervosa" (McCullagh and Tupper, 1940). Others—more numerous—say that hypopituitarism may occur in anorexia nervosa. Of these, some consider the hypopituitarism the result of inanition (Decourt and Michard, 1949 ; Stephens, 1941 ; Loeper and Sainton, 1937) ; others regard the hypopituitarism as of psychological or emotional origin (Sheldon, 1937 ; Spence, 1939 ; Querido, 1949 ; Bartels, 1946) ; others, such as Schermann (1947), hold that the anorexia and other psychological changes and the endocrine manifestations are due to disturbance of the pituitary-diencephalic region by factors not fully understood. Sheldon (1939) suggested that "the mechanism adjusting the body to a lowered intake of food . . . acts . . . by a general inhibition of anterior pituitary activity." Hubble (1952) says that malnutrition can affect the formation of anterior pituitary hormones, and psychogenic depression also occurs.

Confusion of Anorexia Nervosa with Simmonds's Disease.—Some writers use expressions which may well have caused confusion. Cornil and Schachter (1939–41) say that "most cases of anorexia nervosa are really more or less attenuated forms of Simmonds's disease." Selye (1947) states that in anorexia nervosa "the fundamental cause of the symptoms is a secondary hypopituitarism." Decourt (1942) remarks that anorexia nervosa and Simmonds's disease differ only in their original causes. Repetto, Ianni, and Benzecry (1947) report a case as changing from anorexia nervosa into Simmonds's disease, with amenorrhoea signalling the change. Pagniez (1939) thought that anorexia nervosa and Simmonds's disease were distinct, but that there occurred intermediary forms of doubtful nature.

It is proper to emphasize that anorexia nervosa is a disease of psychological origin, while Simmonds's disease is the result of destruction of the pituitary. This is a basic and essential difference, which might be expected to settle the problem, if only at necropsy. Although Escamilla and Lisser (1942), in an exhaustive analysis of the literature on Simmonds's disease, found themselves obliged to distinguish two small exceptional groups of cases (one with pituitary destruction but no typical clinical picture, the other with the typical clinical picture but an apparently normal pituitary at necropsy), many authors seem to think that as a rule there should be no confusion clinically or pathologically. It seems wrong to say, for example, that "anorexia nervosa is an attenuated form of Simmonds's disease" ; and it seems advisable that such expressions as "functional Simmonds's disease" be avoided. (Gull's original name of "hysteric apepsia" (1868) is nearer the truth ; nearer even than "anorexia nervosa," for, in his detailed description (1874), Gull said he thought anorexia nervosa was due to "a failure of the powers of the gastric branches of the pneumogastric nerve.")

Can Inanition Cause Hypopituitarism ?—There remains the question of whether or not hypopituitarism may arise in the course of anorexia nervosa as a result of inanition. It is certain that such hypopituitarism, if it does occur, is reversible, for most patients recover more or less completely when they resume a normal diet after simple persuasion or other forms of psychotherapy (Bond, 1949 ; Brosin, 1941 ; Clow, 1932 ; Hurst, 1939 ; Lorand, 1943 ; Palmer, 1939 ; Pardee, 1941 ; Parker, 1940 ; Roch and Monnier, 1941 ; Venables, 1930).

Evidence from Inanition in Man.—Berkman (1930, 1948), studying the Mayo Clinic's cases from 1917 onwards, said in the later paper that the laboratory tests showed severe insufficiency of the anterior pituitary function. Querido (1949) took a similar view on the basis of the hormone analyses he had gathered from the literature. Davis (1939) found that endocrine therapy was ineffective ; but that does not prove there was no endocrine hypofunction.

The evidence from human starvation is conflicting. Harris (1919) quotes Rubinato (1914) as saying that in pellagra the chromophil cells of the

pituitary are atrophied and crowded together (by which he might have meant they were degranulated), but he also quotes Rossi (1913) to the effect that the pituitary in pellagra is normal. Lhermitte (1944), studying elderly people with deficiency diseases who died with spontaneous hypoglycaemia, found a reduction of the glandular cells of the anterior pituitary; most of the remaining cells were chromophobes, acidophils and basophils being found only here and there; bands of dense fibrosis traversed the anterior lobe. Nicolaief (quoted by Loeper and Sainton, 1937, unfortunately without a full reference) found in victims of the Kiev famine that the eosinophils were reduced in number. Two fatal cases of anorexia nervosa were found to have normal pituitaries (Brosin and Apfelbach, 1941; Osgood, 1938); Decourt and Michard (1949) stated that in fatal anorexia nervosa the pituitary may be normal. Cameron (1947) held the same view.

The Pituitary in Anorexia Nervosa.—It is of interest to review the pituitary changes found in fatal cases of anorexia nervosa. If one may assume that (1) the few authors who have made two reports have not reported the same case twice, (2) all the cases are truly cases of anorexia nervosa—and most of them are, (3) following Sheldon (who said (1939) that Kylin's monograph on Simmonds's disease is "really one of the more important papers on anorexia nervosa"), all Kylin's cases (1935, 1937) are cases of anorexia nervosa, then there have been collected about 440 cases of that condition, in the course of this study. Of these 29 died, and 22 came to necropsy. There is no mention of the pituitary in 14 of these reports, but in the other eight the histology is given. It is not easy to know what to make of them, partly because technical methods are not given and technique plays a large part in the examination of a pituitary. With such reservations in mind the glandular cells of the anterior pituitary can be described as definitely abnormal in only three of the eight cases: Richardson's case, Kylin's third case (Karin H.), and this case (K. L.).

The Other Endocrines in Human Inanition.—No definite conclusion emerges from the findings in other endocrine glands under pituitary influence. Packard and Wechsler (1934) observed both hypertrophy and degeneration in the adrenals of four people dying of chronic malnutrition. Elliott (1914) reported two fatal cases of anorexia nervosa; in one the adrenals were "crammed with lipoid," in the other normal. McCullagh and Tupper (1940) found normal adrenals in a fatal case of anorexia nervosa. Nicolaief (Loeper and Sainton, 1937) reported atrophy of thyroid and adrenals, and lack of follicle ripening in the ovaries. As to the gonads and their function, it is sometimes thought (Repetto *et al.*, 1947) that amenorrhoea is a sign of hypopituitarism, but the amenorrhoea often comes on early in the disease (Hubble, 1952, and other writers); in the present case it was almost the first event. Sydenham (1946), telling of women prisoners in Japanese hands in Hong Kong in the last war, says that amenorrhoea was the rule during the first few months, but that menstruation was re-established and maintained during three years of semi-starvation. Temporary amenorrhoea following an emotional upset or change of surroundings is very common among adolescents.

Evidence from Inanition in Animals.—A great amount of experimental work on animals has been done, with some bearing on this problem. Degranulation and atrophy of chromophil cells in the pituitary was observed in rats deficient in vitamin E (Barrie, 1937) and in starvation (Jackson, 1917; Werner, 1939; Mulinos and Pomerantz, 1940). Werner (1939) observed loss of pituitary potency in starved rats; Mulinos, Pomerantz, Smelser, and Kurzrok (1939) found that pituitary gonadotrophic hormone abolished inanition anoestrus in rats. Mulinos and Pomerantz (1940) studied malnutrition in 300 albino rats. The changes in the pituitary have already been mentioned; in the adrenal, thyroid, spleen, thymus, liver, and gonads the changes resembled those that follow hypophysectomy. Mulinos and Pomerantz found this so striking that they called malnutrition "pseudo-hypophysectomy." D'Angelo, Gordon, and Charipper (1948) found the adrenals hypertrophied in starved guinea-pigs, and Lockwood and Hartman (1933) found hypertrophy in deficiency of vitamin C and B_1, but atrophy in vitamin A deficiency. Rat adrenals were hypertrophic in vitamin B_2 deficiency (Findlay, 1928).

Conclusion.—The many contradictions between these reports are less surprising when one remembers that different species were being observed, and that malnutrition varied in degree, in nature, and in duration. However, one cannot ignore the positive reports of loss of granules, etc., in the glandular cells of the pituitary in malnutrition, and of hypoplasia of adrenals, gonads, and thyroid, which suggest a diminution of the corresponding pituitary hormones. In the present case the pituitary was remarkable for its lack of nor-

mally granulated cells, and other organs showed many features found in definite hypopituitarism. This can be said, because Sheehan and Summers (1949) have exhaustively analysed the gross and microscopic findings in a large number of reported cases of definite Simmonds's disease. What they have shown to be usual in Simmonds's disease is closely matched in this case of anorexia nervosa; the parallel is close or exact in the ovaries, uterus, adrenals, liver, spleen, lymphoid tissue, kidneys, and thyroid (there is inadvertently no microscopic material from the thyroid, but macroscopically it was "small and pale").

It appears reasonable to conclude, from all the evidence, that in some cases of severe anorexia nervosa the inanition can cause hypopituitarism. It is perhaps not surprising, in view of the complexity of the matter and its many seeming contradictions, that no more clear-cut statement can at present be made.

Summary

A fatal case of anorexia nervosa is reported, with gross and microscopic necropsy findings. The adrenals, gonads, thyroid, and other organs showed atrophic changes comparable to those found in Simmonds's disease.

The pituitary was very cellular. Basophil and chromophobe cells were present in normal proportions, but nearly all other cells were sparsely granulated and normal acidophils were very scanty. There are some reports in the literature of similar pituitary abnormalities in human and animal malnutrition.

Anorexia nervosa and Simmonds's disease are quite distinct from each other, but may be confused because of clinical similarities. The similarities have given rise to the idea that hypopituitarism due to inanition may result from anorexia nervosa. The findings in this case, and in man and animals as reported in the literature, are thought to uphold the conception that this happens in some cases.

I wish to acknowledge my indebtedness to Dr. S. Leonard Simpson, consultant endocrinologist to St. Mary's Hospital, who has not only given me the use of the clinical material but also made many helpful suggestions and criticisms. Thanks are due also to the London Hospital, who gave Dr. Simpson access to their records. Dr. G. E. Harre is also thanked for doing the necropsy and preliminary histological work while she was pathologist at Willesden General Hospital. Dr. C. L. Foster, reader in biology at St. Mary's Hospital Medical School, gave helpful advice. Dr. E. Kawerau, of the Chemical Pathology Laboratory at St. Mary's Hospital, kindly did the sugar estimations on the control post-mortem blood specimens. My chief, Professor W. D. Newcomb, has given me much help and encouragement.

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THE STRUCTURAL FEATURES OF EPILOIA, WITH SPECIAL REFERENCE TO ENDOCARDIAL FIBROELASTOSIS

BY

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While the morbid anatomical features of epiloia are probably its best-known aspect, the condition as a whole is obscure. It is rare, and general pathologists have few opportunities to study it. The object of this communication is to enumerate the lesions of epiloia, and to add another one, fibroelastosis of the heart, to the rather long list of structural abnormalities encountered in this condition.

The material used in the preparation of this paper included four cases at the Fountain Hospital, and, thanks to Dr. J. G. Greenfield and Dr. W. H. McMeneney, I had also access to the collection at the National and the Maida Vale hospitals, and in addition histological sections of cases were lent to me by Dr. R. M. Norman, Dr. W. Thomas Smith, and Dr. A. J. N. Warrack.

The characteristic case of epiloia (Table I) has tuberous sclerosis of the brain manifested by epilepsy with or without mental defect, adenoma sebaceum of the face, and one or more of the abnormalities listed in this table. Adenoma sebaceum may, however, be absent, especially in infancy and early childhood, and incomplete, *formes frustes* of the disease, such as adenoma sebaceum without neurological symptoms, or cases with only visceral lesions, have been described, particularly among the patients' relatives and in the late, adult type of the disease. It should be noted in this connexion that less extensive cerebral lesions need not necessarily give rise to either epilepsy or mental defect, and may easily remain undetected in post-mortem examinations.

The characteristic hard nodules of tuberous sclerosis can be palpated better than seen in the freshly removed brain, but may become more conspicuous in the fixed material after the meninges have been stripped. They are situated in the cerebral hemispheres, vary greatly in number and size, and frequently calcify. Lesions have also been described in the cerebellum, brain-stem, and spinal cord; these are, however, relatively uncommon, The larger nodules are well seen in sections stained by the Nissl method as areas of absent or deficient cortical staining. Corresponding sections stained by any of the glial methods show as areas of dense fibrous gliosis situated mainly in the cortex, but also in the subjacent white matter.

Cortical lamination in the nodules is absent, the number of nerve cells greatly diminished, the remainder being pyknotic, shrunken, sometimes abnormally large and orientated at unusual angles. The number of glial cells, chiefly of astrocyte type, is, on the other hand, increased. Some of them may also be abnormally large, having several nuclei and homogeneous cytoplasm. These are particularly frequent in the gliotic nodules of the white matter and in the more superficial portions of the cortical nodules. The bulk of the substance of the nodules is formed, however, by a dense feltwork of glial fibres. The distribution of the fibrous gliosis is quite uneven and dense patches or tufts of glial fibres are frequently seen upon the background of only moderately gliosed tissue.

"Candle-guttering" is the name given to subependymal nodules projecting into the ventricles—usually the lateral ventricles. They tend to be more cellular than the cortical nodules and it is noteworthy that such frank gliomata—astrocytoma or spongioblastoma—as have been described in epiloia, were also situated near the lateral ventricles, and were therefore probably derived from the nodules of "candle-guttering."

The list of skin lesions in epiloia is, for obvious reasons, longer than that in other organs. The commonest amongst them is adenoma sebaceum, an acneiform, papular, colourless, or brownish rash of the face, which may only appear in later childhood or after puberty, when it often takes on a reddish tinge. The term "adenoma" is unfortunate. The usual microscopical appearances indicate only a moderate increase and a somewhat

TABLE I
EPILOIA

Brain	Skin	Heart	Kidney	Eye	Lungs	Bones	Skeleton	Other Organs
1. **Tuberous sclerosis**	1. **Adenoma sebaceum**	1. **Rhabdomyoma (Purkinjeoma)**	1. **Mixed benign tumours**	1. **Phakoma**	1. Congenital cysts	1. Periosteal nodules	1. Spina bifida	Haemangioma and benign tumours have been mentioned in thyroid, spleen, thymus, breast, duodenum
2. **Candle-guttering**	2. Peau de chagrin	2. Lipoma, fibroma	2. Rare malignant tumours, e.g. carcinoma or sarcoma		2. Alveolar cell adenoma	2. Osteoporosis	2. Syndactyly, polydactyly	
3. Glioma	3. Flat warts	3. Fibroelastosis	3. Polycystic kidney, teratoma			3. Cysts		
4. Secondary changes, e.g., demyelination, softening, etc.	4. Haemangioma 5. Fibroma 6. Subungual fibroma and haemangioma 7. Vitiligo 8. Leucoderma 9. Café au lait spots					4. Melorheostosis 5. Bony surface excrescences		

Rare lesions appear in ordinary type; common lesions in bold.

disordered arrangement of the sebaceous glands, blood vessels, and fibrous tissue in the more superficial portion of the dermis. There is also often hyperkeratosis, thinning of the epidermis, and irregularity in the length of the rete pegs.

Another characteristic skin lesion is " peau de chagrin "—areas of shagreen skin often situated on the buttocks or thighs. They are formed by closely set, flattish, or more elongated outgrowths of the superficial layer of the skin.

The cardiac lesions, " rhabdomyomata," are among the more familiar features of epiloia. They are formed by large cells the cytoplasm of which is sometimes coarsely granular, but more commonly forms radial or tangential fibrils, or bundles of fibrils, leaving much or even most of the intracellular space empty. A strip of cytoplasm in some of these cells may show distinct transverse striation, particularly if stained with Heidenhain's haematoxylin (Fig. 1). These cells are structurally identical with the normal Purkinje's cells of the heart, and their tumour-like accumulation in epiloia may be described more appropriately as " purkinjeoma " rather than the customary " rhabdomyoma." These cells are by no means always arranged in tumour-like nodules. They sometimes lie singly or in small separated groups in the subendocardial layer or within fibrotic areas in the myocardium.

Two of the cases showed congenital fibroelastosis of the heart. The left ventricle was hypertrophied (Fig. 2), some of the chordae tendineae thickened and the endocardium over the mitral aspect of the interventricular septum opaque and thick. Histological examination (Fig. 3) showed a considerable increase in fibrous and elastic tissue throughout the endocardium involving in a few places the subjacent muscle (Fig. 4). Nests of Purkinje cells were sometimes seen within this fibroelastic layer or just under it.

The kidney is one of the most frequently affected organs in epiloia. The structural changes, hamartomata or tumours, vary greatly in size, number, and histological structure. They show frequently a mixed and disorderly arrangement of such structures as fat, blood vessels, muscle, and fibrous tissue, some of which are reminiscent of elements in an embryonic kidney. Many varieties of these malformations and tumours have been described as fibroma, myoma, angioma, endothelioma, lipoma, perithelioma, being present usually in combination with each other. Malignant renal tumours—carcinomata and sarcomata—have also been described. It is worth mentioning that while the histological appearances in some of the tumours described in the literature were certainly suggestive of malignancy, no published records of secondaries in other organs have been found. Professor Dorothy Russell (verbal communication) has, however, seen such a case with secondaries of a lipofibrosarcoma.

Polycystic kidneys and teratomata have also been described in epiloia.

Another lesion in epiloia is the phakoma of the retina, a whitish plaque or nodule which may be

FIG. 1.

FIG. 3.

FIG. 1.—Cells in purkinjeoma showing transverse striation. Heidenhain's haematoxylin ×180.

FIG. 2.—The left ventricle is hypertrophied. The endocardium and some chordae tendineae are thickened and opaque.

FIG. 3.—Numerous elastic fibres are present in the thickened endocardium. Verhoeff's elastic stain ×180.

FIG. 4. — Fibroelastosis of endocardium and subjacent muscle. Verhoeff's elastic stain ×44.

FIG. 2.

FIG. 4.

visible on ophthalmoscopic examination. It may consist of rather uniform, densely arranged glial cells and fibres.

Many other lesions have been described in the numerous case reports of epiloia available in the literature. Some of them have only been mentioned once in single cases, and all are probably rarer than those already enumerated. Amongst them were haemangiomata, cysts, and benign uniform or mixed tumours in such organs as the thyroid, spleen, thymus, breast, and duodenum. The material used in the preparation of this communication included an example of a cystic lung and of a papillary alveolar cell adenoma of the lung.

Discussion

Congenital fibroelastosis of the endocardium has been recently discussed by several workers and the subject has been well reviewed by Gowing (1953). The condition affects infants and young children, is believed to be invariably fatal, and is characterized by progressive heart failure associated with cardiac hypertrophy and endocardial thickening. The association of endocardial fibroelastosis with epiloia has not received any attention, although it was undoubtedly present in at least two of the previously reported cases: in Case 15 of the series reported by Critchley and Earl (1932) and in the case of Norman and Taylor (1940). Two further instances of this association were, as mentioned above, found in the present material. It may well be that its incidence in epiloia, the frequency of which cannot be estimated at present, may prove significant if attention is paid to it in future studies.

Thanks to the kindness of Dr. R. Pugh, sections have been compared with those in the collection of The Hospital for Sick Children, Great Ormond Street. The histological appearance of the endocardium was essentially similar, but there was no involvement of the subendocardial muscle such as was present in my material.

Two further examples of cardiac fibroelastosis associated with other conditions have been seen at the Fountain Hospital: one with gargoylism and the other with dermatomyositis. It would appear, therefore, that fibroelastosis of the heart cannot be regarded as a specific structural change occurring in one disease only, even though there are certain cases in which it is the sole abnormality seen at necropsy.

Summary

Structural abnormalities in epiloia are found in the brain, skin, heart, kidney, lungs, bones and, occasionally, other organs. The common lesions are tuberous sclerosis of the brain, adenoma sebaceum of the face, purkinjeoma (rhabdomyoma) of the heart, renal tumours, and phakoma of the retina. Incomplete forms of the disease are also seen, particularly among the relatives of the patients. Two cases of fibroelastosis of the heart were encountered in the present series, and another two cases had been previously recorded in the literature. This association may prove to be more frequent if attention is paid to it in future studies. Fibroelastosis has also been seen in association with other diseases and cannot therefore be regarded as a specific change occurring in only one disease.

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CEREBRAL MUCORMYCOSIS

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The *Mucoraceae* are generally considered saprophytic, but since Cohnheim (1865) described two cases of pulmonary mycosis due to mucor a considerable number of infections of ear, lungs, and stomach by this fungus have been reported. Affections of the meninges and brain, however, are somewhat rare. After the paper of Paltauf (1885) no further reports appeared till Gregory, Golden, and Haymaker (1943) reviewed the literature and presented three cases of their own occurring in moribund diabetics. Subsequently LeCompte and Meissner (1947) and Wolf and Cowen (1949) added one case each. In the report of Hafström, Sjöqvist, and Henschen (1941) their diagnosis cannot be accepted from the illustrations or the description.

The case presented here differs from those previously reported, as the infection occurred in the absence of diabetes and without involvement of the orbit.

Case Report

M.R., a boy of 5 months, was admitted on November 25, 1952. He had been in all respects normal until three weeks before admission, when he had a mild upper respiratory infection as well as diarrhoea with blood and mucus in the stools. He appeared to respond favourably to treatment, but six days before admission he began to vomit. Frank haematuria was noticed the next day and puffiness of the face a day later. From then on the infant had difficulty and pain in passing progressively scantier amounts of urine. Three days before admission he had a convulsion affecting the right half of the body.

On admission he was pale and oedematous. His weight was 7 kg., pulse 128, respirations 40, temperature 99.2° F., blood pressure 105/75 mm. of mercury. Physical signs were normal in the major systems. Haemoglobin was 6.8 g. %, and red blood cells 2,440,000 per c.mm. Films showed anisocytosis and poikilocytosis, hypochromia and polychromasia, occasional nucleated red cells and myelocytes. The infant was Group A rhesus positive. The blood urea level was 340 mg. %, and the blood cholesterol 180 mg. %. The urine was acid with 780 mg. % protein, and no reducing substance. The deposit contained numerous erythrocytes, leucocytes, and occasional granular casts.

Treatment for the first 24 hours was by oral fluids. As the anuria persisted the full Bull regime (Bull, Joekes, and Lowe, 1949), 350 ml. of peanut-oil-glucose mixture given daily by pernasal intragastric drip, was begun on November 26 and continued till December 3. The output of urine during this period ranged from 10 ml. to 85 ml. per day. Investigations on November 28 showed 1,800 mg. % urinary protein, 400 mg. % blood urea, and haemoglobin 6.5 g. %. The next day 50 ml. of compatible packed red cells was transfused. As signs and symptoms of respiratory infection appeared, 250,000 units of penicillin daily were given intramuscularly. By December 2 urinary proteins were 840 mg. % and the blood urea 410 mg. %. Next day a brisk attack of diarrhoea caused severe dehydration necessitating a subcutaneous infusion of 300 ml. of Hartman's solution. The pernasal intragastric drip was resumed on December 4, but, even with adequate doses of chloromycetin, the diarrhoea recurred on the next day, and 360 ml. of Hartman's solution was given intravenously. By December 8 marked oedema had appeared and that night respirations became rapid and deep. Blood urea was 488 mg. %. On the morning of December 9 the infant passed three black stools and vomited altered blood. He died at 5.30 p.m. that day.

Necropsy

Necropsy was performed 21 hours after death. The body was that of an oedematous male infant.

The respiratory system showed some aspirated vomit in the trachea and the bronchi of the upper lobe of the right lung. The liver weighed 210 g., and its cut surface revealed a firm, rather yellow, fatty parenchyma. The cardiovasular system was normal. The gall bladder, spleen, pancreas, and adrenals were macroscopically normal. Both kidneys appeared slightly enlarged (weight 65 g.) and showed numerous congested vessels on the surfaces. The capsules stripped easily, leaving smooth, pale subcapsular surfaces. Sections showed a normal cortico-medullary pattern except for numerous points of congestion. The rest of the genito-urinary tract was normal. Numerous punched-out erosions were present in the mucosa of the stomach, while the rest of the alimentary

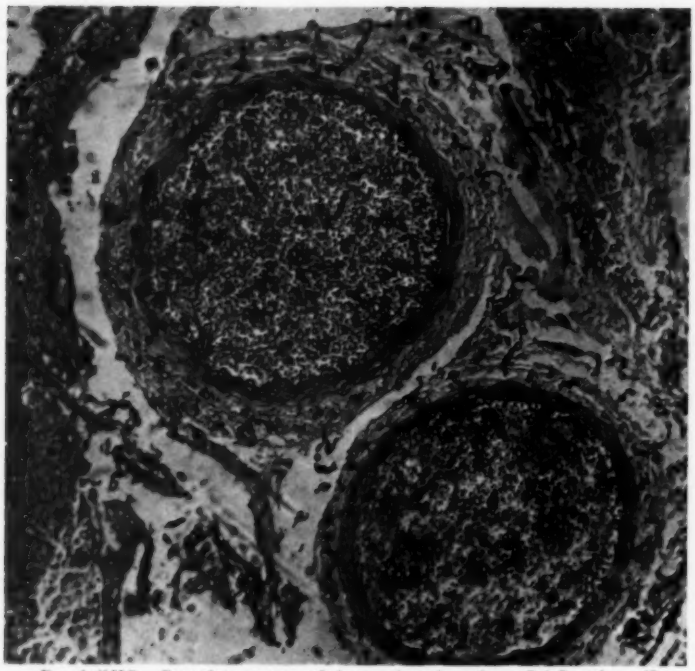

FIG. 1 (1534).—General appearance of the vessels and meninges. P.A.S. stain. ×48.

tract appeared normal but contained much altered blood. The skull and dura appeared normal. The orbital surface of the left frontal lobe showed a peculiar, pinkish brown, sharply demarcated area about 6 × 4 cm. As the significance of this was not realized, the brain was, as is our usual practice, fixed whole in formol-saline. The middle ears and orbits seemed normal.

Histology

Blocks taken from both kidneys and from the discoloured area of the brain were dehydrated in alcohols, cleared in benzene, and embedded in paraffin wax. Sections of the kidneys showed marked exudative and proliferative glomerulitis with the formation of epithelial crescents in many places.

Brain sections were stained by Harris's haematoxylin and eosin, Verhoeff's elastin and Van Gieson's mixture, the Gram-Weigert technique for fibrin, Mallory's phosphotungstic acid haematoxylin, Gram's stain, Ziehl-Neelsen, and by the periodic acid-Schiff technique.

The most striking feature was a thin layer of haemorrhagic exudate in the leptomeninges over the orbital surface of the frontal lobe and accompanying vessels in the sulci (Fig. 1). The exudate consisted of a loose fibrin network enmeshing numerous erythrocytes and a variable number of granulocytes, lymphocytes, and large round cells. Varying degrees of degeneration affected these cells. Much more prominent than the cells were considerable amounts of large, branching, non-septate hyphae (Fig. 2). They were considered the agent responsible for the tissue reaction. The hyphae were found also among the muscle fibres of arterial walls and in the lumina of arteries and veins. In the arterial wall they appeared to penetrate in the ground substance between fibres, eventually lifting the internal elastic membrane off the muscle, stretching and finally rupturing it (Figs. 3 and 4). Loose fibrin thrombi containing large amounts of mycelium and variable numbers of leucocytes filled the vascular lumina. Capillaries contained predominantly polymorphonuclear cell collections without evidence of fibrin whether hyphae were present or not.

The grey matter adjoining the meningitic areas showed oedema, some cellular infiltration by round cells and occasional granulocytes. The latter were usually related to hyphae although more often the fungus was present without any cellular reaction (Fig. 5). No obvious lesions were seen in the white matter. Gliosis could not be demonstrated in relation to the fungus.

The mycelium stained well with haematoxylin and by the P.A.S. method ; it was Gram-negative and failed to retain carbol-fuchsin in the Ziehl-Neelsen stain.

Discussion

The condition was not recognized at necropsy and no cultures were put up. Absolute proof of the identity of the fungus could not, therefore, be obtained. However, the appearance, and in particular the intravascular growth, compares closely

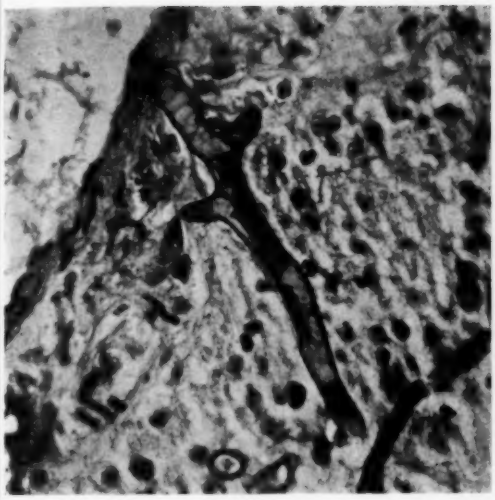

FIG. 2

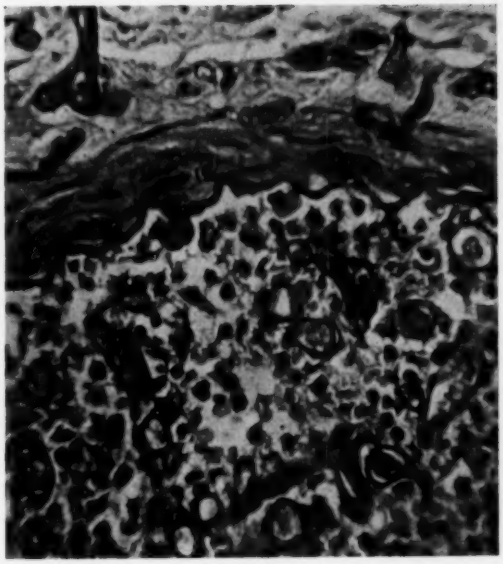

FIG. 3

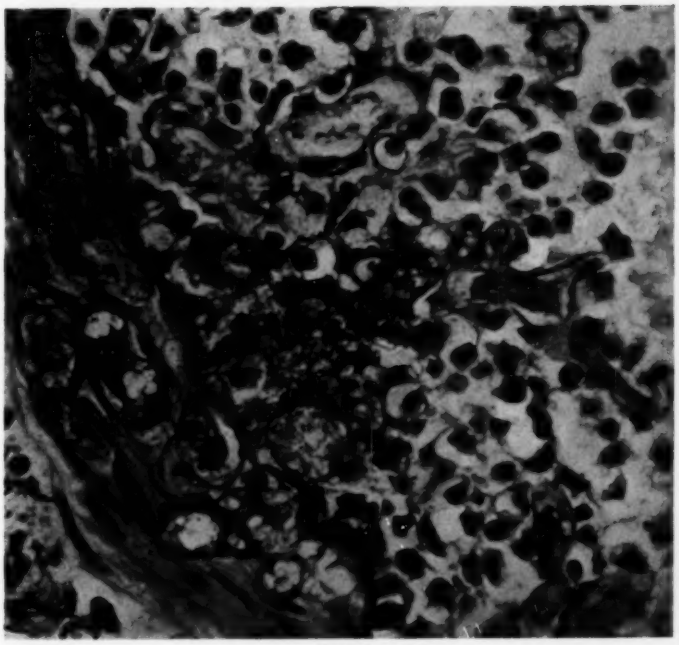

FIG. 4

FIG. 2 (1537).—Non-septate branching mycelium. Haematoxylin and eosin. ×192.

FIG. 3 (1535). — Mycelium within and without an artery. P.A.S. stain. ×192.

FIG. 4.—Another photomicrograph showing mycelium in an arterial wall with special reference to the internal elastic lumina.

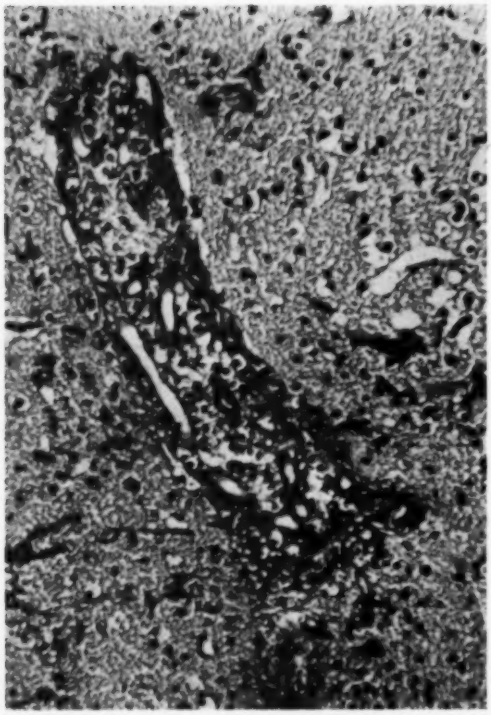

FIG. 5. (1540).—The relatively slight reaction around a fairly severely involved small vessel in cortex. Haematoxylin and eosin. ×150.

with the descriptions and illustrations in the previous papers and in *The Pathology of Tropical Diseases* by Ash and Spitz (1945).

Sections were submitted to Dr. R. W. Riddell, of the Brompton Hospital, London, who reported as follows: "The nonsegmented and very wide mycelium is typical of the *Mucoraceae*."

It seems likely that the infection occurred in the terminal phase of uraemia and that the long indwelling nasal tube of the intragastric drip was instrumental in introducing and spreading the fungus. In the absence of clinical orbital infection, the ethmoid, olfactory plate, or the nasal veins must be regarded as the most likely pathways.

Summary

A case of mucormycosis of the central nervous system is reported. It occurred in an infant aged 5 months dying in uraemia due to acute nephritis. Unlike the previously reported cases, there was no coexistent diabetes, and no frank invasion of the eye and orbit. As far as can be ascertained, this is only the seventh case of this condition to be reported.

I wish to thank Professor D. S. Russell and Dr. R. W. Riddell for their advice and helpful criticism. I am indebted to Mr. J. G. Williamson, of the Birmingham Children's Hospital, for the photographs. The sections were prepared by Mr. G. Haworth.

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FURTHER EXPERIENCES WITH THE MALE-TOAD PREGNANCY TEST USING EUROPEAN SPECIES

BY

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(RECEIVED FOR PUBLICATION SEPTEMBER 19, 1953)

The satisfactory reports on the Galli Mainini male toad test for pregnancy which have been published from nearly every part of the world (Cowie, 1948 ; Bhaduri, 1951) show that the test can be performed on species of toads or frogs indigenous to the district where the laboratory is situated. In England and Europe the toads *Bufo bufo* (syn. *B. vulgaris*) and *B. calamita* and the frog *Rana esculenta* are used for the test. Favourable results with these species have been reported by Hinglais and Hinglais (1948), Haines and Ferreira (1949), Klopper and Frank (1949), Frazer and Wohlzogen (1949), and Bach, Szmuk, Robert, and Klinger (1949). In a later paper Frazer and Wohlzogen (1951) described a method for estimating urinary gonadotrophin using *B. bufo*.

Modifications of the Earlier Technique

The principle and the technique of the test which have already been described for a South American species (Haines, 1948) are readily adapted to European species and the technical details of modifications in procedure which we found necessary for the new species have been described by Law (1949).

The toads available weighed only 15–25 g. and the volume of urine injected was reduced to 2 ml., i.e. 1 ml. per 10 g. body weight. Because there was an injection mortality of up to 40% a modified purification method was tried (Scott, 1940) and thereafter no deaths occurred from injections. Brody (1949) adopted a similar method with good results. This procedure, by concentrating the hormone, increases the sensitivity of the test, and these advantages outweigh any loss of time spent in the elution process.

The test is performed (by tradition) on an early morning specimen of urine, but positive results are obtainable on specimens collected at other times. Quantitative tests are performed on untreated urine diluted with distilled water. The patient's serum (2 ml.) instead of urine gives consistently good results. It seems preferable to use serum for dilution tests and doubtful cases because the concentration of hormone in the urine may fluctuate, depending on various factors, including the rate of urinary output.

Reading of the Test

The end-point is taken as the appearance of spermatozoa in a specimen of toad's urine usually collected from the cloaca. Spermatozoa may be found in less than one hour after injection. Our tests were read at the end of three hours—or four if negative at three. Under circumstances where a weak positive response was to be expected (see below) readings were taken at five and six hours. A weak positive is denoted by a delayed response of the test animal and by the presence of relatively few sperms per low-power field. Increasing numbers of sperms may be found at the end of the fourth and fifth hours when only two or three per field were found at the end of the third hour. In our experience few sperms per field, in the absence of a delayed response, indicate exhaustion of the test animal. The urine of positive reactors was found clear of spermatozoa within two days of the injection. All animals used in the series were tested (and found negative) before a test was begun.

A series of 1,023 male toad tests using this technique was carried out between April, 1949, and May, 1950. This report is based upon the experimental series augmented by information collected from the routine use of the test in the three years following.

Results in Pregnancy

Routine pregnancy tests were carried out on 732 samples of urine. There were 238 negative results : six of these may be designated false negative. All provided positive results after the first negative. Five subsequently aborted at or before the twelfth week and the sixth case went to term. There were no false results among 494 positive tests. All of these were confirmed clinically.

ROUTINE PREGNANCY TESTS

	Weeks after Missed Period				Total
	1st	2nd	3–6	7–14	
Positive tests ..	32	70	314	78	494
Negative ,, ..	14	32	121	71	238

Most of the tests (59.4%) were done about three to six weeks after the first missed period, but there were 102 positives in the first two weeks. The earliest confirmed positive response was obtained two days after the period was due in a patient admitted to hospital for colporrhaphy. All six false negative results were obtained in the groups three to six weeks after the missed period.

Controls

Control tests were made on 40 gynaecological and obstetrical patients. Negative controls were taken on 20 patients who were in hospital for a diagnostic curettage which excluded normal pregnancy. Positive controls were provided by 20 patients attending ante-natal clinics from the twentieth to the twenty-fourth weeks of pregnancy. Toads were given injections of proprietary gonadotrophins in 68 instances. It was found that freshly caught toads would respond to doses of 5 to 10 i.u. It required 20 to 25 i.u. to evoke a response in those kept in captivity and used frequently throughout the summer. Even this loss of sensitivity did not interfere with the reliability of the test in routine work.

A small number of tests were run in parallel with other species, viz., *Bufo arenarum*, *Xenopus laevis* (female), and *Rana esculenta*.

CONTROL TESTS USING OTHER SPECIES

No. of Tests	Control Animals	Results	*Bufo bufo* Results
36	*B. arenarum*	35 correct 1 false negative	36 correct
62	*X. laevis* (female)	59 correct 3 false negatives	62 correct
12	*R. esculenta*	12 correct	11 correct 1 false negative

Ectopic Pregnancy

The following interesting results were obtained in 30 patients admitted to hospital because of suspected ectopic pregnancy:

Final Diagnosis	Positive	Negative
Ectopic pregnancies	11	2
Normal	4	—
Follicular cysts	—	3
Salpingitis	—	2
Corpus luteum haematoma ..	—	1
Nil found	—	7

The final diagnosis was made either at laparotomy or by examination under anaesthesia. In 11 of the 13 true ectopic gestations the male toad test was positive. Its value in this condition lies in its rapidity. It can usually be completed between admission and operation. A negative reaction does not exclude this diagnosis but indicates, in agreement with the clinical findings, that the pregnancy has been interrupted (Ascheim, 1950).

Threatened Abortion

In this group there were 19 patients who gave negative results and abortion followed in each case. Positive results were obtained in 25 patients. In some of these a weak positive response (see above) was obtained and this indicated a poor prognosis. Abortion within seven to 14 days was the rule. But we are not yet able to form a reliable opinion in every case of this kind because we have no means of measuring accurately the absolute "weakness" or "strength" of a test and the number of cases examined is too small.

Other Results

Four tests were performed in one fatal case of chorion carcinoma and positive results were obtained up to a dilution of 1 in 500.

Negative tests were given on four samples of urine from two patients with testicular teratoma. More than 100 tests were made on eight cases of hydatidiform mole. Before evacuation of the mole the titre was found to be not less than 1 in 200 and as high as 1 in 600 in one case. Normal pregnancies never yielded a positive test in dilutions greater than 1 in 100. In normal pregnancy the test is usually negative within 100 hours of complete expulsion of the placenta. In three patients the tests remained positive for two, five, and eight months respectively following evacuation of a mole and in the apparent absence of intra-uterine chorionic tissue as judged by repeated negative curettage. Dilution tests were consistently negative. Two cases of syncytial endometritis (syncytioma) gave negative results. One case of acute hydramnios at 16 weeks' gestation gave a positive result in a dilution of only 1 in 50 and a positive test to a titre of 1 in 150 was recorded in one case of hyperemesis gravidarum. Positive results were obtained in six samples of maternal blood while the results were negative using corresponding samples of foetal cord blood.

Discussion

The indigenous species of male toad *B. bufo* has proved to be an eminently satisfactory test animal. Using the method of concentrating the urinary gonadotrophin we have achieved a degree of accuracy (99.2%) comparable with all other standard pregnancy tests. This agrees with the findings of Frazer and Wohlzogen (1950). The toad is not

subject to vagaries of spermatogenesis of the common frog *R. temporaria* (Cei, 1944) or of the male *Xenopus* as observed by Robbins and Parker (1948) and by Schwabacher (1951). Neither does it yield false positive tests when injected with distilled water, adrenaline, jaundiced urine, or urine containing an excess of F.S.H. as reported in different species of frogs.

False negative results in pregnancy tests are often attributed to performing the test too early in the pregnancy when the urinary gonadotrophin level would be low or to the variation of hormone levels in individual pregnancies (Robbins and Parker, 1948). The high incidence of false negatives recorded by Klopper and Frank (1949) and by Diczfalusy and Westman (1950) can probably be so attributed. Our false negatives occurred between the third and sixth week of pregnancy and five of these patients, though free from symptoms when the test was performed, subsequently aborted, suggesting that gonadotrophin excretion is irregular in these unstable pregnancies.

The rapidity of the test is particularly useful in assisting the diagnosis of ectopic pregnancy where early operation may be indicated. A result may be had in two or three hours, and in our small group 11 out of 13 true ectopics gave a correct positive result.

In cases of hydatidiform mole the test is applicable to diagnosis and follow-up. Many observations may be made—for example, in one case weekly tests were carried out over a period of eight months with very little trouble and expense. The diagnostic titre for both moles and chorion epitheliomas appears to be about 1:200, though many molar pregnancies will be encountered in which the hormonal concentration is considerably less. Syncytioma sometimes confused clinically and histologically with chorion carcinoma invariably yields negative tests. This agrees with the observation that chorionic gonadotrophin is elaborated by the cytotrophoblast (Wislocki, Dempsey, and Fawcett, 1948).

We have found the test cheap, simple to perform, accurate and rapid, and therefore particularly useful in the study of abnormalities of pregnancy as well as in routine pregnancy diagnosis.

We are grateful to members of the clinical staff of Chelsea Hospital for Women and to many others for submitting specimens for the test and for making available the clinical data of their patients.

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A STRAIN OF STAPHYLOCOCCUS RESISTANT TO FIVE ANTIBIOTICS

BY

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(RECEIVED FOR PUBLICATION OCTOBER 20, 1953)

Since the use of antibiotics in the treatment of staphylococcal infections has become established, the emergence of resistant strains has been frequently reported. The incidence of penicillin resistance has been described by Barber and Rozwadowska-Dowzenko (1948), Barber, Hayhoe, and Whitehead (1949), Rountree and Thomson (1949), and by Cairns and Summers (1950). Organisms resistant to penicillin and streptomycin isolated from two cases of meningitis have also been described (Anderson and Ellis, 1951). While testing the sensitivity of freshly isolated strains of pathogenic bacteria to terramycin and other antibiotics, Ransmeier, Brown, and Davis (1951) found four strains of *Staph. pyogenes* which were resistant to penicillin, streptomycin, chloramphenicol, aureomycin, and terramycin. Clarke, Dalgleish, and Gillespie (1952) report the appearance and dissemination in hospital of staphylococci showing resistance to these five antibiotics and also to sulphadiazine.

Changes which take place in staphylococci developing resistance *in vivo* to penicillin have been reported by Spink, Ferris, and Vivino (1944), who showed that such resistance was often accompanied by increased susceptibility to the bactericidal action of whole blood, and possibly to other defence mechanisms. Klimek, Cavallito, and Bailey (1948), studying a strain made resistant to penicillin *in vitro*, found that at a level of 1 mg. per ml. the fermentation reactions of the organism were almost completely suppressed. Pigmentation was first reduced and then completely lost; haemolysis was retarded and the coagulase reaction was negative in the single test conducted. Bellamy and Klimek (1948) describe variants which were resistant to 1–4 mg. of penicillin per ml. and had lost the ability to produce acid from lactose, sucrose, maltose, and mannitol. They would not grow in the presence of 6.5% sodium chloride nor would they reduce nitrates.

A strain of staphylococcus recently isolated in these laboratories from a cerebral abscess proved to be resistant to five antibiotics and also to sulphadiazine. The organism was so atypical on primary isolation that a detailed study of it was made in an attempt to discover what changes might be associated with multiple resistance to antibiotics. The clinical history of the strain and the investigations made are now considered in detail.

Clinical History

The patient, J.A.D., a man aged 46 years, was admitted to the Maudsley Hospital, London, on August 12, 1953, with a provisional diagnosis of cerebral abscess. For 24 hours before admission he had been given penicillin, 1 mega unit four-hourly, and sulphadimidine, 3 g. six-hourly. While in hospital, he received systemic penicillin, 1 mega unit six-hourly for five days, and streptomycin 1 g. b.d. also for five days. Aureomycin was given orally in a dosage of 1 g. b.d. for four days and three doses of chloramphenicol to a total of 3 g. were given at eight-hourly intervals just before death on August 17. Chloramphenicol, 2 mg., and 50 mg. of streptomycin were instilled into the left and right ventricles through frontal burr-holes. At necropsy a right temporo-sphenoidal abscess was found. There was no obvious pathology in any of the systems to account for this. Pus from the abscess was taken for bacteriological examination. Gram-stained films showed large numbers of Gram-positive cocci, and culture yielded a growth of a staphylococcus.

Colonial Morphology and Growth Characteristics

On primary isolation on aerobic horse blood agar, the colonies were somewhat smaller than normal, but the general contour and surface texture did not present any unusual features. The colonies lacked pigment, and even scraping up the growth failed to demonstrate it. Plates kept at room temperature for seven days did not show any significant pigment. There was no haemolysis of the surrounding medium. The growth was

viscid and stringy in consistency but emulsified well in saline, and there was no auto-agglutination. Gram-stained films showed a Gram-positive coccus of normal morphology, size, and arrangement.

Growth in nutrient broth produced a uniform turbidity with a stringy deposit which could be dispersed by shaking. Turbidimetric studies showed that the rate of growth in broth was considerably slower than that of the Oxford staphylococcus under similar conditions. The organism grew well on nutrient agar, and in gelatin stab cultures produced fully developed saccate liquefaction in seven days at room temperature. It grew in the presence of 6.5% sodium chloride, but rather more slowly than a control strain.

Biochemical Reactions

Five sugars were inoculated and examined at the end of seven days' incubation at 37° C. The organism produced acid in glucose, lactose, and saccharose but failed to ferment maltose or mannitol. In litmus, milk acid and clot were formed after 48 hours' incubation. The methyl red test and the Voges-Proskauer reaction were both positive. Nitrates were reduced and ammonia was formed. Neither indole nor hydrogen sulphide was produced.

Production of Coagulase

The organism was first tested on the slide by the method of Cadness-Graves, Williams, Harper, and Miles (1943). Only slight granularity of the emulsion could be produced compared with the massive clumping of a control strain. Different samples of plasma gave identical results. Repeated sub-culture on blood agar resulted in some increase in clumping, but the reaction remained less vigorous than that given by a control organism.

This led to a more detailed investigation of the coagulase-forming properties of the strain and a tube test was set up in the following manner. Samples of citrated human and rabbit plasma were diluted 1 in 5 and 1 in 10 with both nutrient broth and with saline. Tubes containing 1 ml. of these plasma dilutions were inoculated with 0.1 ml. of an 18-hour culture of the organism. Duplicate tubes were inoculated with a known coagulase producer and a further rack of plasma dilutions acted as a control. All racks were incubated at 37° C. and negative tubes were left overnight before being finally recorded. The result of this experiment is summarized in Table I.

TABLE I
COAGULASE ACTIVITY OF STRAIN J.A.D. AND POSITIVE CONTROL

	Rabbit Plasma				Human Plasma			
	Dilution in Broth		Dilution in Saline		Dilution in Broth		Dilution in Saline	
	1:5	1:10	1:5	1:10	1:5	1:10	1:5	1:10
Strain J.A.D.	−	−	−	−	−	−	−	−
Positive control	+ (40 min.)	+ (40 (min.)	+ (40 min.)	+ (40 min.)	+ (40 min.)	+ (40 min.)	+ (40 min.)	+ (40 min.)
Plasma controls	−	−	−	−	−	−	−	−

+ = clot formation. The times taken for clot formation are shown in parentheses.

Toxin Production

The strain was examined for haemolysin production by the plate method described by Elek and Levy (1950a). Repeated tests showed that only the δ toxin was produced. Further tests were performed using the double diffusion gradient principle (Elek and Levy, 1950b). The strain was found to produce only two antigen-antibody flocculation lines after 48 hours' incubation in an atmosphere of air plus 30% CO_2.

Animal Pathogenicity

A rabbit was injected intravenously with 0.5 ml. of an 18-hour broth culture. At the end of seven days the animal still appeared well and was sacrificed. Necropsy showed no abscess in any of the internal organs and the spleen was of normal size. Pathogenicity was further assessed using the mouse as a test animal. The intramuscular method of Selbie and Simon (1952) and the intraperitoneal method of Christie, North, and Parkin (1946) were both employed. The former technique, which provides finely graduated readings, showed an increase of only 1 mm. in the diameter of the injected thigh after the first day. Subsequent measurements failed to demonstrate any difference between the control and the test limbs. In the intraperitoneal method of Christie *et al.*, 1 ml. of the standard bacterial suspension was injected into three mice. No deaths occurred in the 48 hours following injection.

Antibiotic Sensitivity

The staphylococcus was first screened for sensitivity by the plate method using compressed antibiotic tablets (Evans Medical Supplies Ltd.). It was evident by this method that the strain was resistant to penicillin, streptomycin, chloramphenicol

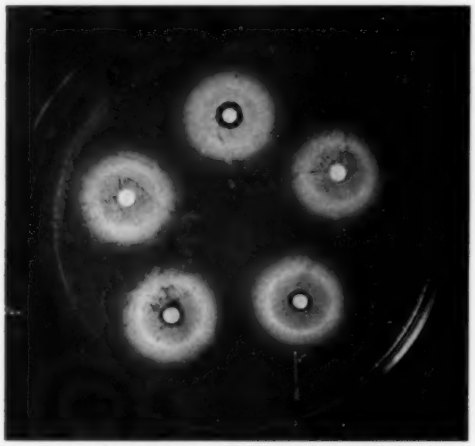

FIG. 1.—Antibiotic spectrum of strain J.A.D. Antibiotics from the top disc, clockwise, are chloramphenicol, penicillin, terramycin, aureomycin, and streptomycin.

col, aureomycin, and terramycin (Fig. 1). Sulphonamide sensitivity tests were performed by the method of Harper and Cawston (1945). Growth occurred in the presence of 10 mg. of sulphadiazine per 100 ml. of medium. The results of the tube titrations using serial dilutions of antibiotics are seen in Table II.

TABLE II

TUBE TITRATIONS OF ANTIBIOTIC SENSITIVITY OF STRAIN J.A.D.

Antibiotic	Inhibitory Concentration (μg./ml.)
Penicillin	25
Streptomycin	>100
Chloramphenicol	100
Terramycin	50
Aureomycin	100

Production of Penicillinase

To three concentrations of penicillin in nutrient broth 1 ml. of a six-hour broth culture of the organism was added. The tubes were incubated with uninoculated duplicates for two hours at 37° C. This time was chosen since it was felt that penicillinase production might be slower with an atypical strain. Samples from each tube were transferred to Heatley cylinders embedded in a plate inoculated with the Oxford staphylococcus. The result of subsequent incubation is shown in Fig. 2.

The quantitative penicillinase production was kindly estimated by Dr. Patrick Collard using an iodometric method described by Perret (1953), and

FIG. 2.—Penicillinase production by strain J.A.D. The top three control cylinders contain 1 unit, 5 units, and 10 units of penicillin per ml. The lower cylinders contain similar concentrations plus the test organism.

1 ml. of a six-hour broth culture of the organism was found to destroy 640 units of penicillin in one hour.

Phage Type

Dr. R. E. O. Williams reported that the organism was not lysed by any of the standard filtrates in use at that time.

Discussion

Some of the changes described in strains of staphylococci made highly resistant to penicillin *in vitro* seem capable of appearing in organisms clinically resistant to several antibiotics. Of these changes, the loss of pigment and diminished production of coagulase seem the most likely to give rise to difficulty in identification. In this particular case, the slide coagulase test was equivocal and, had the tube test alone been performed, the organism might well have been discarded. Fisk (1940) and Duncan and Walker (1942) have drawn attention to the optimum ratio between organism and plasma. However, this staphylococcus appeared to produce insufficient extracellular coagulase to cause clotting when transferred to any of the plasma dilutions and the slide test gave the only clue to the possible identity of the organism.

The absence of α-toxin, the results of an animal inoculation, and the failure to ferment mannitol (Knott and Blaikley, 1944) may all be taken as evidence of reduced pathogenicity and serve to confirm the observations of Spink *et al.* (1944) that

such organisms may be more susceptible to the body defences. It seems unlikely that an organism with these properties could cause a cerebral abscess in man. Elek and Levy (1950a), studying various haemolysin combinations in staphylococci, found that of the strains producing δ toxin alone, only 2% were human pathogens, while 39% were coagulase-positive skin strains. Investigations performed on this strain would appear to confirm the observations of Howard (1954), who found that the number of diffusible antigen-antibody flocculation lines were directly related to the relative virulence of the organism for mice. He also found that strains with a simple haemolysin pattern (α or δ) gave significantly fewer flocculation lines than those strains with a more complex pattern (αδ or αβδ).

It is more probable, therefore, that total resistance with its attendant changes developed during the course of treatment, either by adaptation or by the selection of mutants. The patient received all the antibiotics concerned at some time during his illness with the exception of terramycin. However, "cross-resistance" between aureomycin and terramycin is well recognized and has been described by Herrell, Heilman, and Wellman (1950) and by Fusillo and Romansky (1951).

With the increasing use of antibiotics, often in combination, the emergence of staphylococci showing multiple resistance must be expected to occur with greater frequency. The study of this particular strain indicates that the changes described in cocci with artificially induced resistance to penicillin can no longer be regarded merely as phenomena *in vitro*. The normal characteristics necessary for the identification of *Staph. pyogenes* fail to apply to such strains and much caution is required in assessing their significance when isolated from pathological material. The apparent loss of pathogenicity in the organism under consideration cannot be regarded with optimism from the clinical point of view, since Clarke *et al.* (1952) have shown that cross-infection was common with their strains which showed multiple and total resistance to antibiotics.

Summary

A strain of staphylococcus isolated from a cerebral abscess showed resistance to five antibiotics and sulphadiazine.

Certain metabolic and biochemical changes were demonstrable which have also been described in strains made highly resistant to penicillin *in vitro*.

Difficulties may be experienced in the identification of such strains isolated from pathological material.

I am indebted to Mr. Murray Falconer, Director, Guy's Maudsley Neuro-Surgical Unit, for permission to publish the details of this case; also to Mr. Peter Schurr, F.R.C.S., and Mr. B. H. Dawson, F.R.C.S., for access to the clinical notes and for their help in many other ways.

I also wish to thank Dr. R. E. O. Williams, who phage-typed the strain, and the Department of Medical Photography, Guy's Hospital, for Figs. 1 and 2.

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A NOTE ON THE SEROLOGY OF SARCOSPORIDIOSIS AND TOXOPLASMOSIS

BY

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Serological methods for the diagnosis of toxoplasmosis have received considerable attention since the first authentic report of a fatal infection in a human infant (Wolf, Cowen, and Paige, 1939). The complement fixation reaction (Warren and Sabin, 1942) and the dye test (Sabin and Feldman, 1948) are now recognized as the two best methods of detecting the disease. Serological studies in sarcosporidiosis have not, however, been given the same amount of attention, although this infection has a much longer history than that of toxoplasmosis. For example, organisms designated as sarcosporidial in nature were described in the laryngeal muscle cells of a human patient (Baraban and Saint Remy) as long ago as 1894. Similar aggregations have since been described in isolated human patients in the muscles of tongue, biceps, heart, etc., and these have usually been given the specific name of *Sarcocystis lindemanni* (Rivolta). The discovery that chronic toxoplasmic infections are often associated with the formation of "pseudocysts" containing large numbers of organisms not only in nervous tissue but in skeletal and heart muscle adds to the suspicion that some reported cases of sarcosporidiosis in man and animals might in fact be cases of toxoplasmosis. Different investigators give different dimensions for these organisms. "Sarcosporidial spores" described in human infections range from 8 to 9 μ long (Baraban and Saint Remy, 1894) to 4.25 μ long (Darling, 1909, 1919). While it seems unlikely that organisms regularly of the first dimensions are *Toxoplasma*, suspicion arises in the case of smaller organisms. The issue is complicated by the fact that in some of these reports the "septa" and enclosing membrane associated with the more typical sarcosporidial cysts could not be demonstrated. Kean and Grocott (1945) concluded that in the absence of inoculation and serological studies the absolute identification of these two parasites is impossible.

In October, 1951, Mühlpfordt used the Sabin-Feldman dye test in an endeavour to differentiate between infections of *Toxoplasma* and *Sarcocystis*. "Miescher's tubes" (sarcosporidial cysts) recovered from gullet muscles of sheep or goats were inoculated per os or intraperitoneally into guinea-pigs, rats, and hamsters, and the serum of these animals was tested by the dye test against toxoplasms. In all cases, preliminary tests made before the inoculation with sarcosporidia produced negative results, but after the infection was allowed to establish itself the animals reacted positively, thus suggesting that antibodies reacting with the toxoplasms were produced by the sarcosporidial infection. Tests were then carried out with the sera of 45 sheep. In 26.6% of the animals the dye test was positive using *Toxoplasma* in the presence of detectable parasites; in the remaining animals the results of both parasitological and serological examinations were negative. From this investigation Mühlpfordt concluded that sarcosporidiosis cannot be differentiated from toxoplasmosis by the dye test.

In view of this apparent non-specificity of the dye test and the difficulties in microscopical diagnosis, it was decided to investigate the possibilities of antigenic relationships between *Sarcocystis* and *Toxoplasma* as far as the dye test and the complement-fixation antibodies were concerned, and to attempt to find a way of differentiating the two diseases.

Methods

Microscopical Examination of Sheep Tissues for Evidence of Sarcocystis tenella Infection.—The sites selected for microscopical examination were the oesophagus, heart, and diaphragm muscles. The oesophagus was the primary site for the large and obvious cysts, while scrapings from the muscle of heart, diaphragm, and oesophagus were placed separately on slides in normal saline under coverslips and examined under the 2/3 objective. In view of the obvious limitations of such methods the term

"undetectable" was used in preference to "negative" when parasites could not be found by these means.

The Complement-fixation Reaction for Toxoplasmosis.—The method used here was exactly that as devised by Warren and Sabin (1942) and as modified by Warren and Russ (1948). The test was already in regular use by one of us in problems of incidence in toxoplasmosis. The antigen was prepared from a chorio-allantoic membrane extract from developing chick embryos inoculated with suspension of *Toxoplasma*.

The Complement-fixation Reaction for Sarcosporidiosis.—This reaction, again, was in current use by one of us in studies on serological methods for the detection of sarcosporidial infections in sheep. The antigen was prepared in the following manner:

Sarcocysts (*Balbiania*) were removed, free of muscle, from the oesophagi of infected sheep, washed in normal saline, and ground with sterile carborundum. Enough normal saline was then added to give a 10% suspension. This was quickly frozen and thawed four times in the methylated spirits bath refrigerator and kept at 4° C. for a minimum of 15 hours. The suspension was then centrifuged at 4,300 r.p.m. for two hours, the supernatant clear fluid removed, and normal saline containing 0.1% merthiolate added to give an ultimate concentration of merthiolate of 1 in 10,000. The extract was stored at −70° C. A control antigen was similarly prepared from sheep heart and diaphragm muscle which failed to show infection either in scrapings or sections stained with haematoxylin and eosin; in addition the complement fixation reaction was negative.

The Dye Test.—The Sabin-Feldman dye test was used as modified by Cathie and Dudgeon (1949).

Investigation

Experiment A: Complement-fixation Reaction for Toxoplasmosis.—Rhesus monkeys, guinea-pigs, and rats were given intraperitoneal inoculations of *Toxoplasma* suspensions in normal saline. The inoculum consisted of 0.5 ml. of a 1 in 10 dilution of peritoneal fluid taken from mice with infections of three days' duration. The animals were bled and sera obtained at periods as indicated in Table I.

Serum from an experimentally infected dog was received from the Royal Veterinary College, London, together with normal serum obtained from the same animal before infection.

One positive human serum was obtained from The Hospital for Sick Children, Great Ormond Street, and four others from the Bernhard-Nocht-Institut, Hamburg.

Normal human, monkey, guinea-pig, and rat sera were used as negative controls.

Complement fixation tests were now carried out with these sera which were used in serial dilutions of 1 in 5, 1 in 10, 1 in 20. The antigen was used at a dilution to give four units of antibody per unit volume of the test (0.11 ml.). Guinea-pig complement was used at 3 M.H.D. per unit volume, and the haemolytic system consisted of a 3% sheep cell suspension with 5 M.H.D. of haemolysin. The short fixation method was used of one hour for the initial incubation and 30 minutes secondary incubation at 37° C. in the water-bath. All test sera were previously inactivated at 56° C. for 30 minutes.

Results

Of the three adult guinea-pigs infected with *Toxoplasma*, two gave positive results on sera collected at periods from three weeks to six weeks,

TABLE I

COMPLEMENT-FIXATION REACTION (C.F.R.) ON *TOXOPLASMA*-IMMUNE SERA USING *TOXOPLASMA* AND *SARCOCYSTIS* ANTIGENS

Case No.	Species and Sex	Inoculation Route and Period between Inoculation and Bleeding	Passage in Mice	Diagnosis			
				Microscopic	C.F.R. using *Toxoplasma* Antigen	C.F.R. using *Sarcocystis* Antigen	Dye Test
1	Adult ♂ guinea-pig	I/P 22 days	+	+	−	−	+
2	„ ♂ „	I/P 30 „	+	+	+	−	+
3	„ ♀ „	I/P 39 „	+	+	+	−	+
4	Two normal stock ♂ guinea-pigs			−	−	−	−
5	Adult ♂ Rhesus monkey with infection of *Plasmodium cynomolgi* (14.5.53)				−	−	−
6	Adult ♀ Rhesus monkey	I/P 45 days and 176 days			±	−	+
7	Three young ♂ Rhesus from normal stock				−	−	+
8	Two adult ♀ albino rats	I/P 31 days	−	−	+	−	−
9	„ „ ♂ „ „	I/P 35 „	−	−	−	−	−
			−	−	±	−	+
			−	−	+	−	+
10	Three normal ♂ stock rats		−	−	−	−	−
11	Normal dog	Bled before infection			−	−	−
12	*Toxoplasma*-infected dog	I/M (also S/C 14 days later) 46 days			+	−	+
13	Five normal human sera				−	−	−
14	Five human *Toxoplasma*-immune sera				+	−	+

S/C = Subcutaneous. I/P = Intraperitoneal. I/M = Intramuscular.

as shown in Table I. In all these animals toxoplasms were recovered by passage of tissues into laboratory white mice. The normal stock guinea-pigs returned negative tests.

The Rhesus monkey immune serum was collected six weeks and six months after the initial infection, and gave a strong positive reaction at six weeks and negative at six months. The sera from three normal stock monkeys and that from one monkey previously infected with *Plasmodium cynomolgi* were all negative.

The rats were bled one month following inoculation; two only gave a positive test, one was doubtful, and the fourth negative. In no case could the parasites be recovered by animal inoculation.

Serum from the dog taken before infection was negative, while that obtained after the infection was established was positive.

The human sera from The Hospital for Sick Children and from Hamburg were positive to the test; the "normal" sera were negative.

All the *Toxoplasma*-immune sera were tested using the dye test. The results shown in Table I indicate a good agreement except for one rat and one guinea-pig which developed cytoplasm-modifying antibody but remained negative to the complement-fixation reaction. In Case 6 both complement-fixation reaction and dye test were positive at six weeks, but the dye test only at six months.

Experiment B: Complement-fixation Reaction for Sarcosporidiosis.—An adult male guinea-pig was injected by the intraperitoneal route with 1.0 ml. of sarcocyst antigen. After one week the same dose was repeated and serum collected the week following. This serum was used as a positive control against which the antigen was titrated.

Normal guinea-pig serum was obtained for use as a negative control.

Forty-five sheep sera and tissues to be used in microscopic examination were collected from the abattoir.

Serum was obtained from a reported human case (Pugh, 1950).

Sera were taken from six young lambs 7–8 weeks old.

Antigen titration was performed by the typical "chess-board" method. The results obtained showed the most favourable dilution to be used in the test proper as 1 in 40. The unit volume per tube was again 0.11 ml.; the haemolytic system, a 3% suspension in saline of washed sheep cells with 5 M.H.D. of haemolytic serum prepared by using equal volumes of a 6% cell suspension and saline containing 10 M.H.D. haemolytic doses and incubated at 37° C. for 30 minutes. The complement was used in the tests to give 3 M.H.D. per unit volume and the short fixation method. All sera were previously inactivated at 56° C. for 30 minutes and used in dilutions of 1 in 5, 1 in 10, and 1 in 20.

An identical experiment was carried out on the control positive serum, but using a 1 in 40 dilution of the control antigen.

Results

The 45 sheep tested yielded 36 positive complement-fixation reaction results. Examination of Tables II and III shows that of these 36 positive sheep, 31 possessed parasites, 26 of which were detected by microscopical means, and five showed macroscopic oesophageal cysts. Five were serologically positive in the absence of detectable parasites.

TABLE II

COMPLEMENT-FIXATION REACTION (C.F.R.) ON SHEEP SERA USING *SARCOCYSTIS* AND *TOXOPLASMA* ANTIGENS

No. of Animals	Origin	Age	Presence of *Sarcocystis*	No. Positive to C.F.R. with *Sarcocystis* Antigen	No. Positive to C.F.R. with *Toxoplasma* Antigen
20	Devonshire	3–5 years	16 positive 4 negative	12 1	0 0
20	Somerset	3–4 „	14 positive 6 negative	12 2	0 1
5	Kent	Old cast ewes	5 positive	5	0
6	Experiment	7–8 weeks	6 negative	0	0

Nine sheep of the 45 gave a negative complement-fixation reaction. Of these nine, four possessed parasites shown by microscopical means, while the remaining five sheep were negative both parasitologically and serologically.

Experiment C: Complement-fixation Reaction on Sheep and Experimental Sarcocystis-immune Sera.—This was an exact repetition of Experiment B, but the *Sarcocystis* antigen was replaced by a *Toxoplasma* antigen.

Results

Among the 45 sheep tested as above in experiment B, only No. 28 gave a definite positive result, while No. 23 was doubtful. All other sera gave negative results as shown in Tables II and III.

Experiment D: Complement-fixation Reaction on Toxoplasma-immune Sera.—This was an exact duplication of Experiment A, but the *Toxoplasma* antigen had been replaced by a *Sarcocystis* one.

Results

All sera gave negative results as shown in Table I.

Experiment E: Sabin-Feldman Dye Test on Sheep Sera and Human Sarcocystis-immune and Guinea-pig-immune Serum.—The dye-test was carried out on the sera of sheep Nos. 21–40. Of these, 12 had been shown positive both to the complement-fixation reaction for sarcosporidiosis and to microscopic examination, four serologically only, two microscopically only, and two negative to both tests for sarcosporidia.

TABLE III

SHEEP SERA, A CASE OF SARCOSPORIDIOSIS, AND AN ARTIFICIALLY IMMUNIZED GUINEA-PIG TESTED BY THE COMPLEMENT-FIXATION REACTION USING *SARCOCYSTIS* AND *TOXOPLASMA* ANTIGENS AND THE DYE TEST

No. of Animals	Origin	Presence of *Sarcocystis*	No. Positive to C.F.R. with *Sarcocystis* Antigen	No. Positive to C.F.R. with *Toxoplasma* Antigen	No. Positive to Dye Test
20	Somerset	14 positive	12	0	7
		6 negative	2	1	3
5	Kent	5 positive	5	0	1
6	Experiment	6 negative	0	0	0
1	Human adult	1 positive	1	0	0
1	Guinea-pig artificially inoculated with *Sarcocystis* antigen	—	1	0	0

The test was similarly carried out on the sheep 41–45 which possessed large oesophageal cysts, also on the six lambs which were negative to serological examination, and finally on the human serum which had proved positive to the complement-fixation reaction for sarcosporidiosis, and the control positive serum.

Results

Of the 20 sheep from Somerset, 14 were positive microscopically and six negative; of the 14 sheep positive microscopically, seven gave positive results. Among the six negative to microscopical examination, three gave positive results.

Of the five sheep from Kent with macroscopic cysts, only one gave a positive result. All the lambs as well as the human serum were negative. The correlation of all these results with those of other experiments is clearly indicated in Table III.

Discussion

In certain cases microscopical examination alone is not considered sufficient to differentiate between sarcosporidiosis and toxoplasmosis, therefore serological tests together with animal inoculations must form an important part in distinguishing between infections due to these parasites.

The reliability of the complement-fixation reaction and dye test for the diagnosis of *Toxoplasma* infections is shown, also that the complement fixation reaction is a reliable means of detecting sarcosporidial infections, and that this test is preferable to the dye test, as applied by Mühlpfordt, for detection of this infection in sheep. For example, the five sheep which had macroscopic evidence of infection gave positive complement-fixation reaction results, whereas only one positive and one doubtful were recorded when using the dye test. Again, among the 14 sheep showing microscopic evidence of *Sarcosporidia* 12 gave positive results for the complement-fixation reaction, while only seven did so for the dye test. Of the 31 sera tested by the dye test for sarcosporidiosis, only 14 agreed with the complement-fixation reaction findings; on three occasions the dye test recorded a positive reaction on sera from sheep with undemonstrable parasites where the complement-fixation reaction failed to do so, but, on the other hand, the dye test was negative 10 times when microscopical and complement-fixation reaction examinations were in agreement. Finally the human serum from the case that had been diagnosed as a sarcosporidial infection by examination of sections proved positive to the complement-fixation reaction for sarcosporidiosis, negative to the complement-fixation reaction for toxoplasmosis, and negative also to the dye test.

An analysis of these results had led us to the opinion that, although the dye test gives positive results in some cases of sarcosporidiosis, it is not sufficiently reliable to be used as a means of diagnosing this infection in sheep. On the other hand, these non-specific reactions render the dye test equally unsuitable for the differential diagnosis of sarcosporidiosis and toxoplasmosis.

Experiments C and D, however, indicate that, as far as the complement-fixation reaction is concerned, no demonstrable antigenic relationship exists between the two organisms *Toxoplasma* and *Sarcocystis*.

Conclusions

The dye test gave positive results with *Toxoplasma* sera; also with some *Sarcocystis* sera.

The complement-fixation reaction using *Toxoplasma* antigen gave positive results with *Toxoplasma* sera but negative with *Sarcocystis* sera.

The complement-fixation reaction using *Sarcocystis* antigen gave positive results with *Sarcocystis* sera but negative with *Toxoplasma* sera.

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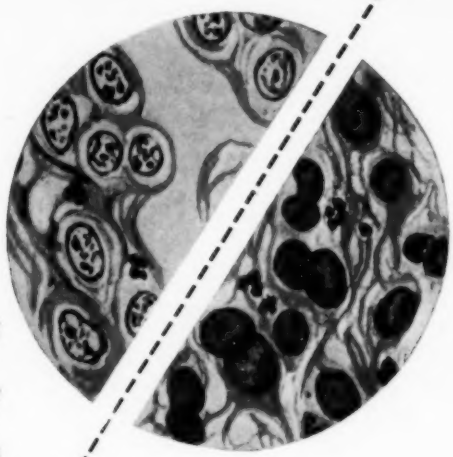

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STREPTOMYCIN-DEPENDENT VARIANTS OF *BACT. COLI* ISOLATED FROM PATIENTS DURING TREATMENT WITH STREPTOMYCIN

BY

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(RECEIVED FOR PUBLICATION AUGUST 17, 1953)

Since the publication in 1947 of Miller and Bohnhoff's report on streptomycin-dependent meningococci, streptomycin-dependent variants of many other bacterial species have been isolated and studied *in vitro* (Paine and Finland, 1948; Kushnick, Randles, Gray, and Birkeland, 1947; Yegian, Budd, and Vanderlinde, 1949). Miller and Bohnhoff also showed streptomycin-dependence *in vivo* when mice infected with streptomycin-dependent meningococci survived unless they were treated with streptomycin.

Reports on the development of streptomycin-dependent variants observed clinically seem to have been relatively scanty. Miller and Bohnhoff found streptomycin-dependent bacteria in the throats of patients who had been treated with streptomycin, but these microorganisms did not play any role in the pathogenesis of the patients' illnesses. Dufourt, Brun, Viallier, and Kalb (1951) found streptomycin-dependent tubercle bacilli in patients receiving streptomycin treatment and claimed that continued administration of streptomycin in certain cases aggravated the infection.

Eight cases of infection of the urinary tract treated with streptomycin are reported. During the treatment bacterial flora emerged which were only able to multiply on media containing streptomycin, i.e., the cultures were completely streptomycin dependent. After the withdrawal of streptomycin the streptomycin-dependent bacteria disappeared. In one patient renewed administration of streptomycin resulted in aggravation of the infection. This case will be described in detail together with a brief account of the seven other cases.

Personal Investigations

Case 1.—A woman, aged 78, who had suffered from recurrent cystitis for many years, was admitted to hospital on August 29, 1952, with a diagnosis of encephalomalacia and cystitis. On admission she complained of burning micturition. Microscopic examination of the urine showed a few leucocytes, and urinary cultures yielded growth of numerous colonies of Gram-negative, lactose-fermenting rods with a sensitivity (determined by the paper-disc method (K. A. Jensen)) to streptomycin +++, sulphathiazole 0, aureomycin +, chloromycetin +, terramycin ++. Dihydrostreptomycin, 0.5 g., was administered four times daily for four days. On the day when streptomycin was withdrawn urinary cultures yielded growth of numerous colonies of Gram-negative lactose-fermenting rods, which only grew on that particular part of the "resistance plate" where the streptomycin disc had been placed. When the bacteria from the resistance plate and from the urine were seeded on agar-plates with and without streptomycin growth only occurred on the plates containing streptomycin. The urine contained a larger number of leucocytes than at the institution of treatment, even though the symptoms had subsided.

The urine was then studied daily, and for 11 days in succession only such bacteria which were able to grow on streptomycin-containing media were found. After that time the flora changed and now chiefly consisted of streptomycin-sensitive bacteria, although a few colonies of streptomycin-dependent organisms could still be isolated. This phenomenon was easily recognized on the resistance plate. On the part where the streptomycin was deposited there was a broad zone of inhibition in the area in which a few colonies grew, and when these were transferred to agar they did not form colonies unless streptomycin was added to the medium.

By way of experiment, the patient was then again given streptomycin, 0.25 g., four times daily for one day. Five hours after the first streptomycin injection the bacterial flora had changed, and now again consisted exclusively of streptomycin-dependent bacteria. The next day the urine contained 50–100 leucocytes per field, which was interpreted as an activation of the infection, although no subjective symptoms developed. For six days the bacterial flora were streptomycin-dependent and then changed once more, so that it now mainly consisted of streptomycin-sensitive bacteria and only a few streptomycin-dependent organisms.

* Lady Tata memorial scholar.

A previous examination of the faecal flora for streptomycin-dependent bacteria had been negative. In order, if possible, to provoke a change in the faecal flora, the patient was given an oral dose of 1 g. of streptomycin daily for three days. Twenty-four hours after the first oral dose the urinary flora had again become streptomycin dependent, and the number of leucocytes in the urine had increased very considerably, myriads of leucocytes per field being present, which had not previously been observed. At the same time, the patient complained of increased frequency of micturition, but there was no pain on micturition. In the faeces the number of coliform bacteria showed a marked decrease during the oral streptomycin treatment, but it was not possible to isolate streptomycin-dependent organisms. This seems to indicate that some factor in the urine was of importance in the change of the bacterial flora to streptomycin dependence.

The patient was discharged and returned for follow-up examination three months later. During the interval there had been no difficulty in micturition. The urine still contained Gram-negative, lactose-fermenting rods, but these were now streptomycin resistant, and no streptomycin-dependent organisms could be isolated.

This case illustrates the fact that flora of streptomycin-dependent bacteria may be found in the urine when urinary infections caused by streptomycin-sensitive organisms are treated with streptomycin. It also shows that the infection may be aggravated when streptomycin treatment is continued. This is in agreement with results from animal experiments performed with the strain which had been isolated in the above-mentioned case. Mice infected with the strain did not die unless they were treated with streptomycin (Stenderup, 1954).

It is remarkable that the urine contained completely streptomycin-dependent bacteria for as long as 11 days after streptomycin had been withdrawn, and at a time when no measurable amounts of streptomycin were detectable in the urine. However, when the dependent strain was transferred to plain broth and incubated at 37° C., it did not change its streptomycin dependence, but remained alive in the broth for eight days after which time the broth became sterile.

Case 2.—A woman, aged 55, who had had several acute attacks of urinary infection during the past 15 years, was admitted to hospital on July 23, 1948, with a diagnosis of chronic pyelonephritis caused by streptomycin-sensitive, lactose-fermenting, Gram-negative rods. Streptomycin was administered, 500 mg., four times a day during the first 24 hours, followed by 0.3 g. daily for the next five days. Four days later the urinary cultures showed that the bacteria had all become completely streptomycin dependent. Two days after the withdrawal of streptomycin cultures yielded growth of Gram-negative, lactose-fermenting rods, which were not dependent on streptomycin, but had become sensitive to streptomycin.

Case 3.—A woman, aged 32, without any past history of previous urinary infections was admitted to hospital on October 28, 1949, for vaginal bleeding during pregnancy. During the hospital stay a urinary infection developed caused by streptomycin-sensitive, lactose-fermenting, Gram-negative rods. Streptomycin was administered, 2 g., during the first 24 hours, followed by 1 g. daily, given in four doses. During the treatment the fever subsided and the subjective symptoms disappeared. After four days' treatment streptomycin was discontinued and on the same day urinary cultures yielded numerous colonies of Gram-negative, lactose-fermenting rods which were all dependent on streptomycin. Repeated cultures three days later revealed a different bacterial flora. No Gram-negative rods but numerous colonies of Gram-positive cocci were seen.

Case 4.—A woman, aged 50, with a past history of recurrent urinary infections extending over many years was admitted to hospital on April 23, 1950, with a complaint of difficulty in micturition. Urinary cultures yielded growth of streptomycin-sensitive, lactose-fermenting, Gram-negative rods for which reason streptomycin was administered, 0.5 g., thrice daily for six days. Urinary cultures performed on the day when streptomycin was withdrawn yielded growth of numerous colonies of Gram-negative, lactose-fermenting rods, which were all streptomycin dependent. Three days later urinary cultures showed innumerable colonies of Gram-negative, lactose-fermenting rods which were now resistant to streptomycin.

Case 5.—A man, aged 74, for seven years had had recurrent urinary infections. The patient was admitted to hospital on January 21, 1951, in an acute attack caused by streptomycin-sensitive, lactose-fermenting, Gram-negative rods. The patient was treated with streptomycin, 1 g. daily, for five days, when renewed urinary cultures showed lactose-fermenting rods which were now streptomycin dependent. Streptomycin was then withdrawn, but after the lapse of another five days there was still growth of streptomycin-dependent, Gram-negative rods and Gram-positive cocci, which were not dependent on streptomycin. As the Gram-positive cocci were sensitive to aureomycin, the patient was treated with this drug, and the urine became sterile. During the streptomycin therapy the patient became symptom-free.

Case 6.—A woman, aged 31, who had had repeated attacks of urinary infections for about 14 months, was admitted to hospital on October 29, 1951, in an acute attack caused by streptomycin-sensitive, lactose-fermenting, Gram-negative rods. The patient was treated with streptomycin, which resulted in a fall in the temperature and improvement of the general condition. Renewed urinary cultures after eight days'

treatment showed growth of some colonies of lactose-fermenting rods which were streptomycin dependent, and the same was the case two days after withdrawal of streptomycin. The patient was discharged and not observed for the next two months when the urine contained streptomycin-resistant, lactose-fermenting, Gram-negative rods.

Case 7.—A woman, aged 50, with a past history of good health, except for "renal gravel" when a child, was admitted to hospital on November 20, 1951, with fever and arthritic pain.

The patient was treated with penicillin and later with aureomycin without any definite beneficial effect. Four days after the discontinuance of the aureomycin therapy the patient began to complain of burning micturition, and the urine, which had previously been normal, now revealed numerous leucocytes and Gram-negative rods which were streptomycin sensitive. Streptomycin was then administered, 0.5 g., four times daily for eight days. During the treatment the urinary symptoms subsided, but in the urine there were still many leucocytes and bacteria, which proved to be Gram-negative, streptomycin-dependent rods. A week after the withdrawal of streptomycin there was still growth of streptomycin-dependent, lactose-fermenting, Gram-negative rods. Then the flora changed to non-lactose-fermenting rods, which were sensitive to chloromycetin, and after treatment with this drug the urine became sterile and the microscopic findings normal.

Case 8.—A woman, aged 37, without any past history of urinary infections, had repeatedly been admitted to hospital with a diagnosis of tuberculous salpingitis and for observation for tuberculous peritonitis. During a hospital stay in 1950 a routine examination of the urine revealed growth of colonies of Gram-negative, lactose-fermenting rods, which showed more abundant growth on a streptomycin-containing medium than on media without streptomycin. The culture was thus only partially dependent on streptomycin and differed from the cultures isolated from Cases 1–7, which did not grow at all when seeded on streptomycin-free media. The patient showed no signs of disease of the urinary tract. At the time when this was disclosed, the patient was under treatment with streptomycin. During the period immediately following the urine was not subjected to renewed examination.

Nine months later an acute attack of cystitis developed; urinary cultures revealed Gram-negative, non-lactose-fermenting rods. These organisms were not dependent on streptomycin, and at that time the patient was not under treatment with streptomycin.

Case 8 shows that streptomycin-dependent bacteria may temporarily emerge in the urine during streptomycin therapy which does not aim at combating a urinary-tract infection.

In Cases 2 to 7 streptomycin-dependent flora emerged in the urine during treatment of urinary infections caused by streptomycin-sensitive bacteria. In contrast to Case 1 it could not be said with certainty that the dependent flora caused an aggravation of the infection, except perhaps in Case 7, in which the urine contained myriads of leucocytes together with the streptomycin-dependent bacteria. Out of regard for the patients streptomycin treatment was not resumed, but it is possible that an aggravation might have occurred if this had been done. At any rate this assumption seems to be in accordance with the results of animal experiments.

When the strains were inoculated intraperitoneally together with mucin into white mice the animals survived, unless they were treated with streptomycin.

In one case (Case 3) the streptomycin-dependent, Gram-negative rods in the urine disappeared completely. In the other cases Gram-negative rods persisted, but changed their behaviour to streptomycin.

In Case 3 the bacteria changed to streptomycin sensitivity, in Cases 1, 4, 6 to streptomycin resistance. This is in accordance with *in vitro* experiments which were performed on the isolated streptomycin-dependent strains. These experiments showed that when a sufficiently large inoculum was seeded in a streptomycin-free medium, growth of bacteria developed. Some of these cultures were resistant, while others were sensitive to streptomycin (Stenderup, 1954).

Summary

Eight cases are reported of infection of the urinary tract in which streptomycin-dependent bacteria developed during treatment with streptomycin.

In all cases (in some cases not until days after withdrawal) the streptomycin-dependent strain disappeared after the withdrawal of the drug. In one case the infection was aggravated when administration of streptomycin was resumed.

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THE USE OF A BILE-AESCULIN MEDIUM AND OF MAXTED'S TECHNIQUE OF LANCEFIELD GROUPING IN THE IDENTIFICATION OF ENTEROCOCCI (GROUP *D* STREPTOCOCCI)

BY

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The name enterococcus, originally given by Thiercelin (1899) to a Gram-positive coccus of faecal origin growing in pairs and short chains, has been widely used for a group of morphologically similar streptococci found in the gut of man and other mammals, in dairy products, and in soil. The limits of this group, formerly rather vague, were satisfactorily defined with the application to it of Lancefield grouping methods by Sherman and his collaborators (Sherman, 1937; Sherman, Mauer, and Stark, 1937; Sherman, Stark, and Mauer, 1937; Sherman, Stark, and Yawger, 1937), and later by Shattock and Mattick (1943) and Shattock (1945 and 1949b). From this work it is apparent that the great majority, if not all, enterococci belong to Lancefield Group *D*, and that in fact this serological property should be taken as the basis of their classification. The group will then include the following species: *Strep. faecalis*, with its varieties, zymogenes, and liquefaciens, *Strep. durans*, and *Strep. bovis*.

The degree of variation in the biological properties of Group *D* streptococci is well shown in Table I reproduced from Shattock (1949b; see also Nyman, 1949).

Table I covers the majority of important biological properties of the group, except bile tolerance, or the ability to grow in the presence of bile in concentrations up to 40% (Weissenbach, 1918), which is usually regarded as common to all species of enterococci, and aesculin hydrolysis.

Attention was drawn to the value of aesculin hydrolysis in the identification of enterococci by Rochaix (1924), and the use of aesculin in bile media was regarded as the best single differential test by Meyer and Schönfeld (1926), who found that 61 of their 62 strains of enterococci hydrolysed aesculin, as compared with only six among 82 strains of *Strep. viridans*. Weatherall and Dible (1929), who obtained similar results, 60 of their 61 bile-tolerant, heat-resistant, mannitol-fermenting diplococci hydrolysing aesculin, considered, however, that the hydrolysis of aesculin in a bile medium was "very little more than an indicator" of bile tolerance. This conclusion was probably responsible for the fact that the test has been little used. The work of Williams and Hirch (1950) suggests, however, that aesculin hydrolysis has an independent value in the classification of bile-tolerant cocci, as only 83.6% of their 140 strains of bile-tolerant streptococci hydrolysed aesculin.

The present paper records an attempt to find a simple, rapid and accurate single procedure for the recognition of enterococci as a group, minor subdivisions of which are of little importance in the routine diagnostic work of a medical laboratory. Results of Lancefield *D* grouping were compared with those of tests for bile tolerance and aesculin hydrolysis, as well as heat resistance, on a number of freshly isolated and stock strains of streptococci.

Source of Material

One hundred and forty-two strains of streptococci and eight strains of micrococci were isolated from human material in the laboratory of a general hospital. The sources of these strains are shown in Table II.

In addition, nine stock strains of *Strep. bovis*, three isolated from human intestine and six from the rumen of the cow, and 12 strains of *Strep. durans* were examined. Of the latter, two were recovered from the human intestines, the remainder being mostly obtained from milk and dairy products.

TABLE I

DIFFERENTIATION OF SPECIES WITHIN GROUP *D* REPRODUCED FROM SHATTOCK (1949a)

Species	Haemolysis	Gelatin Liquefaction	Strong Reduction of Litmus Milk	Mannitol	Sucrose	Raffinose	Survival at 60° C. for 30 min.	Growth	
								at 45° C.	At *p*H=9·6
Strep. faecalis ..	−	−	+	+	±	−	+	+	+
Strep. faecalis, variety zymogenes ..	+	±	±	+	+	−	+	+	+
Strep. faecalis, variety liquefaciens ..	−	+	+	+	+	−	+	+	+
Strep. durans ..	±	−	−	−	−	−	+	±	±
„ *bovis* ..	−	−	−	±	+	+	−	+	−

± = Varies from strain to strain.

TABLE II

SOURCES OF STRAINS ISOLATED IN THE HOSPITAL LABORATORY

Sources	Presumptive Enterococci	Presumptive *Strep. viridans*	Micrococci	Total Numbers of Strains
Faeces ..	65	1	8	74
Urine	38	0	0	38
Vagina ..	10	0	0	10
Wounds, abscesses, etc.	5	0	0	5
Blood cultures	2	0	0	2
Sputum ..	1	0	0	1
Throat ..	0	20	0	20
Total ..	121	21	8	150

Methods of Isolation

One hundred and thirty strains of Gram-positive cocci were selected on the basis of their colonial morphology on McConkey medium. They appeared as small, discrete colonies, most of which were lactose fermenting. Microscopical examination of broth cultures showed eight of these strains to be micrococci and one to be a long-chained streptococcus. The remaining 121 strains, which grew in pairs or short chains, have, for convenience, been referred to throughout this paper as "presumptive enterococci."

To contrast with these, 20 strains of alpha-haemolytic streptococci were isolated from the throats of healthy persons. These strains have been classed with the alpha-haemolytic long-chained streptococcus of faecal origin as "presumptive *Strep. viridans*."

Methods of Testing

All strains were examined by the following tests: Lancefield grouping, heat-resistance, bile-tolerance, aesculin-hydrolysis.

Sixty-eight strains were also tested for fermentation of mannitol and the ability to grow in nutrient broth containing 6.5% NaCl, on blood-agar containing 0.06% potassium tellurite, and in milk containing 0.1% methylene blue, with the decolorization of the dye.

Lancefield Grouping.—(1) The hot-HCl-hydrolysis technique of Lancefield (1933) was employed throughout, using the sediment from 10 ml. of overnight glucose-broth cultures.

(2) All freshly isolated strains (150) were moreover grouped by a modification of the enzyme method of Maxted (1948). The enzyme was prepared by freezing and thawing an agar culture of *Streptomyces albus*. The fluid collected after thawing, which contains the enzyme, was stored without preservative at a temperature below 5° C.; under these conditions its activity was retained for some months. Seitz filtration and *p*H adjustment were omitted on Mr. W. R. Maxted's advice. The streptococci were grown in 10 ml. of glucose broth, or beef-digest broth, for 18 hours. After centrifuging, the supernatant fluid was discarded, and the deposit placed for two minutes in boiling water. After cooling, 0.5 ml. of the proteolytic extract was added and the mixture incubated at 50° C. overnight. As previously noted by Maxted (1948), Group *D* streptococci were found rather resistant to the action of the enzyme, and even after this prolonged incubation the lysis was usually incomplete, making recentrifugation necessary. Satisfactory bacterial extracts were also obtained from enzyme-treated saline suspensions of growth on blood-agar plates.

Streptococcal extracts prepared by both Lancefield's and Maxted's methods were tested in the same way by layering on to grouping serum (obtained from Messrs. Burroughs Wellcome, Ltd.) in glass tubes of 1 mm. internal diameter. The results were read during the following 30 minutes.

Heat-resistance Test.—Approximately 1 ml. of an 18 to 24 hours' broth culture was placed in a 60° C. water-bath; a loopful was removed for subculture on blood-agar after 15, 30, 45, and 60 minutes. Strains surviving this temperature for 30 minutes were termed heat-resistant (Orla-Jensen, 1919; Dible, 1921).

Bile-tolerance Test.—Heavy inocula were made on segments of a nutrient-agar plate containing 5% horse serum, 0.1% aesculin, 0.05% ferric citrate, and sufficient "difco" dehydrated ox-gall powder to give a final concentration corresponding to 40% bile (Williams and Hirch, 1950). The plates were examined for growth after 24 hours' incubation.

Hydrolysis of Aesculin.—This property was tested on the previously described bile-aesculin plate. Strains which hydrolysed aesculin produced a black colour in and immediately around the colonies. Bile-tolerant organisms which did not hydrolyse aesculin gave rise to whitish colonies. The plates were incubated for 24 hours; growth for a longer period was found not only unnecessary but also undesirable as it was liable to obscure the results, when as many as 12 strains were cultured on one plate. As Weatherall and Dible (1929) have pointed out, this is not truly a test of the capacity of an organism to hydrolyse aesculin, since strains which are not bile-tolerant do not grow on this medium and are, therefore, not tested.

Results

The results are summarized in Tables III and IV.

Of the 121 "presumptive enterococci," 117 belonged to Lancefield Group *D*. All these Group *D* strains were heat-resistant, bile-tolerant, and all hydrolysed aesculin. Of the four strains which did not react with Group *D* serum, two were heat-resistant but intolerant to bile, and two were neither heat-resistant nor bile-tolerant. These four strains were alpha-haemolytic.

None of the 21 strains of "presumptive *Strep. viridans*" reacted with Group *D* serum and 19 of them were neither heat-resistant nor bile-tolerant; two strains grew on bile-aesculin plates without hydrolysing aesculin, but neither was heat-resistant.

None of eight strains of micrococci was heat-resistant and none hydrolysed aesculin, but six were bile-tolerant.

All *Strep. bovis* and *Strep. durans* strains were bile-tolerant and hydrolysed aesculin, their extracts reacted with Group *D* serum without concentration. All strains of *Strep. durans* were, moreover, heat-resistant, but only four out of the nine strains of *Strep. bovis* survived 60° C. for 30 minutes.

Table IV shows the results of the subsidiary four tests, mannitol fermentation and growth in media containing 6.5% NaCl, or 0.06% potassium tellurite, and in milk containing 0.1% methylene blue, performed on 68 of the above 117 Group *D*, heat-resistant, bile-tolerant, aesculin hydrolysing strains. Only 60 (88.3%) strains gave positive results with all four tests.

All cocci (150 strains), with the exception of stock strains of *Strep. bovis* and *Strep. durans*, were grouped by both Maxted's and Lancefield's methods. Both techniques gave identical precipitation results with Group *D* sera in all cases except one; this strain gave a positive Group *D* reaction on acid extraction but consistently negative results when extracted by Maxted's method.

TABLE III

RESULTS OF TESTS ON STRAINS OF ENTEROCOCCI

Total No. of Strains Tested	No. of Strains with Common Properties	Lancefield Group *D*	Heat Resistance	Bile Tolerance	Aesculin Hydrolysis
Presumptive enterococci 121	117	+	+	+	+
	2	−	+	−	−
	2	−	−	−	−
Micrococci 8 ..	6	−	−	+	−
	2	−	−	−	−
Presumptive *Strep. viridans* 21	2	−	−	+	−
	19	−	−	−	−
Strep. bovis 9 ..	4	+	+	+	+
	5	+	−	+	+
Strep. durans 12 ..	12	+	+	+	+

TABLE IV

RESULTS OF ADDITIONAL TESTS ON 68 STRAINS OF ENTEROCOCCI

		Growth in Medium Containing 6·5% NaCl	Fermentation of Mannitol	Growth on Plate Containing 0·06% Pot. Tellurite	Growth in Medium Containing 0·1% Methylene Blue
Numbers and percentage of strains	Positive	66 (97%)	67 (98·5%)	64 (94%)	63 (93%)
	Negative	2	1	4	5

Discussion

All 138 Group *D* strains, in this survey, and none outside this group were found to possess the property of aesculin hydrolysis. Bile tolerance, although equally constant within the group, was shared by some (six out of 33) strains outside it, and is therefore less specific for enterococci. Heat resistance is evidently neither a constant nor exclusive character of Group *D* streptococci, for five out of nine strains of *Strep. bovis* failed to survive 60° C. for 30 minutes, and on the other hand two of the 25 strains of *Strep. viridans* (alpha-haemolytic streptococci not belonging to Group *D*) proved to be heat-resistant.

The behaviour of two strains of alpha-haemolytic, non-Group-*D* streptococci and of six strains of micrococci, which grew on the bile aesculin plate without hydrolysing aesculin, together with the previously quoted figures from Williams and Hirch (1950), point to the value of including aesculin in a bile medium.

The bile-aesculin plate, therefore, appears to be a simple reliable means for the recognition of enterococci. It is much simpler and cheaper than

Lancefield grouping, since as many as 12 strains can be inoculated on one plate.

The following procedure has been adopted in the routine work of this laboratory for the presumptive identification of enterococci. The Gram-positive cocci are subcultured (*a*) in 10 ml. of glucose broth and (*b*) on a bile-aesculin plate. The next day the morphology of the growth in glucose broth is examined in a hanging drop. Strains growing in pairs and/or short chains and hydrolysing aesculin are classified as enterococci. The glucose broth culture is used only for the grouping of those strains which fail to hydrolyse aesculin.

It must be stressed that in this preliminary survey very small numbers of streptococci other than those of Group *D* have been examined. Thus, whereas the property of aesculin hydrolysis in a bile-containing medium has been satisfactorily demonstrated among enterococci, its specificity for the group remains to be proved.

Summary and Conclusions

In an attempt to select a single reliable and simple method for the identification of enterococci (Group *D* streptococci) the following principal tests were employed: Lancefield grouping, heat-resistance, bile-tolerance, and aesculin hydrolysis. One hundred and seventeen Lancefield Group *D* strains were found to be heat-resistant, bile-tolerant, and aesculin-hydrolysing. Of 25 alpha-haemolytic streptococci, not of Group *D*, none hydrolysed aesculin, but two were heat-resistant and two were bile-tolerant. From the very high correlation between Lancefield grouping and hydrolysis of aesculin on a bile-containing medium it has been concluded that the latter is a valid test for the recognition of enterococci, having the advantage of simplicity over the former.

Extracts for Lancefield grouping were prepared by two methods: (1) the hot-HCl technique of Lancefield and (2) the enzymic method of Maxted. Both methods gave equally good results.

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THE ERYTHROCYTE SEDIMENTATION RATE AND THE PLASMA VISCOSITY

BY

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Certain factors are common to the erythrocyte sedimentation rate and the plasma viscosity. Thus fibrinogen increases the erythrocyte sedimentation rate (Gram, 1921; Greisheimer, Johnson, and Ryan, 1929) and Hardwicke, Ricketts, and Squire (1950) found that a direct logarithmic relation exists between the erythrocyte sedimentation rate and the plasma fibrinogen concentration. Also fibrinogen exerts the greatest effect of the plasma protein fractions, weight for weight, on the plasma viscosity (Naegeli, 1931). Globulin increases the sedimentation rate but not to the same extent as does fibrinogen, weight for weight (Ley, 1922; Linzenmeier, 1923; Fåhraeus, 1929); similarly, globulin exerts a greater effect than albumin and a lesser effect than fibrinogen, weight for weight, on the plasma viscosity. That the plasma viscosity is affected mainly by the plasma proteins and only slightly influenced by the non-protein constituents is known (Naegeli, 1931; Cowan and Harkness, 1947), and Miller and Whittington (1942) state that the plasma viscosity can be taken as an index of the power of the plasma to cause red cell aggregation and rouleaux formation. Whittington (1942) was able to correlate the maximum erythrocyte sedimentation rate with the plasma viscosity and the whole blood viscosity. (This latter value is affected by the degree of the red cell aggregation.) Thus the plasma proteins have a significant effect on both the erythrocyte sedimentation rate and the plasma viscosity. Many hydrophilic colloids besides plasma are capable of causing red cell aggregation with increased rates of sedimentation; these include gum acacia, gelatin, agar agar, and casein solutions (Fåhraeus, 1929), and Hardwicke and Squire (1952) showed that the concentrations of macromolecules, such as fibrinogen, dextrans, gelatin, gum acacia, and polyvinyl pyrrolidone bore a linear relation to the maximum erythrocyte sedimentation rate. Also, they showed that the degree of the effect on the maximum erythrocyte sedimentation rate varied with the size of the macromolecules.

Comparing the maximum erythrocyte sedimentation rate of various blood samples, at a constant haematocrit value of 45%, with their corresponding plasma viscosity values, it was found that out of 99 results, 96 showed a direct correlation between the maximum erythrocyte sedimentation rates and the plasma viscosity values, but three blood samples with very high plasma viscosity values had much slower maximum erythrocyte sedimentation rates than was expected. The possible reasons for this disparity were examined.

Methods

A. Blood, 10 ml., was taken from each of 99 patients and normal controls into 0.1 ml. of a solution containing 6 g. ammonium oxalate and 4 g. potassium oxalate in 100 ml. (Lawrence, 1950). The haemoglobin level, packed cell volume, and mean corpuscular haemoglobin concentration were estimated. Then in 55 samples the maximum erythrocyte sedimentation rates were observed in a 200 mm. column of 2–5 mm. internal bore, by plotting the fall of the red cell columns every three minutes. The maximum rates of fall were corrected from the chart of Rourke and Ernstene (1930) to correspond to the maximum rates of fall at a packed cell volume of 45%. In the remaining 44 samples, the packed cell volume was adjusted to 45% by removing or adding plasma, and the maximum erythrocyte sedimentation rates were derived by plotting the fall of the red cell columns every three minutes.

The plasma specific gravity was estimated (Harrison, 1947) and finally the plasma viscosity was estimated at 25° C. by the method described by Lawrence (1950).

From the data obtained, namely, the whole blood haemoglobin concentration, the packed cell volume, and the plasma specific gravity, the red cell specific gravity was calculated, using the formula of Phillips *et al.* (1945) quoted by Harrison (1947).

B. Samples of blood, each of 20 ml., were taken from a normal person into 0.2 ml. of the oxalate mixture used in experiment A. After washing twice in the final suspending fluid, the red cells were resuspended at a packed cell volume of 45% in various

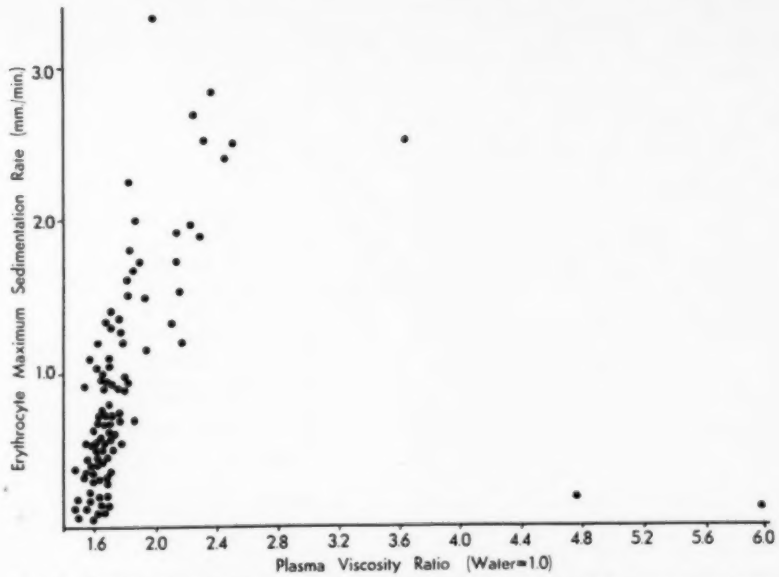

FIG. 1.—The maximum sedimentation rate of 99 samples of blood plotted against their corresponding plasma viscosity ratios. The packed cell volume was adjusted in each case to 45%

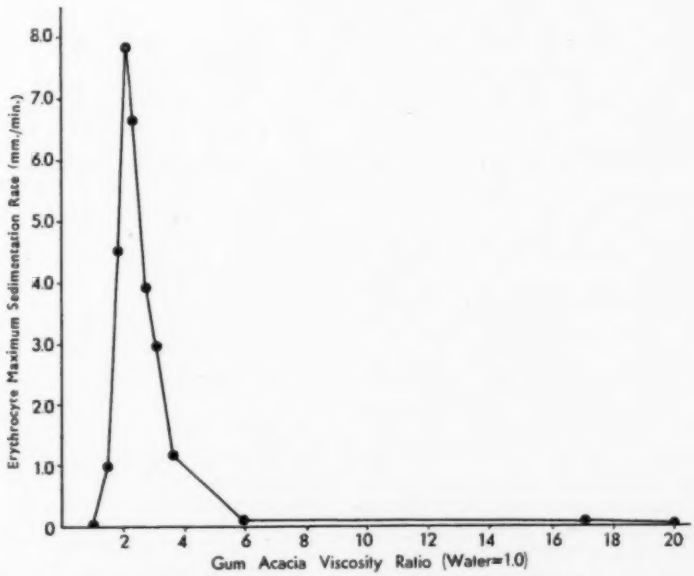

FIG. 2.—The maximum sedimentation rate of red cells suspended in various dilutions of gum acacia at a packed cell volume of 45%.

dilutions of (*a*) gum acacia in physiological saline, (*b*) polyvinyl pyrrolidone in isotonic solution ("plasmosan," May and Baker), (*c*) dextran in physiological saline ("dextraven," Benger).

These red cell suspensions were left at room temperature for 30 minutes and then the maximum erythrocyte sedimentation rate of each sample was plotted by reading the height of the red cell column in 200 mm. tubes every three minutes.

Results

Of the 99 blood samples shown in Fig. 1 in which the maximum erythrocyte sedimentation rate is plotted against the corresponding plasma viscosity, 96 show an increase of the maximum erythrocyte sedimentation rate corresponding to the increase of the plasma viscosity. No simple relation could be found to exist between these two factors, although an empirical formula was developed.

Thus plasma viscosity $= a + b.\ V$,

where $V = \frac{\text{(maximum erythrocyte sedimentation rate)}}{\text{(specific gravity difference between red cell and plasma)}}$

and the constants ($a = 1.531$, $b = 0.0161$).

The variance about the line is 0.01621. If the variance of the values of the plasma viscosity is the same at all values of V, then 95% of the plasma viscosity ratio values will lie within a range of 0.253 on either side of the line. In other words, the estimation of the plasma viscosity from the maximum erythrocyte sedimentation rate, or vice versa, is only very approximate. It was also found that although variations in specific gravity difference between erythrocyte and plasma was allowed for, it did not appear to increase the precision of the estimation to any great extent.

The results of three blood samples fell well outside the line of the other 96 results in Fig. 1. These three showed a much slower maximum sedimentation rate proportional to the plasma viscosity than the others. All three were samples taken from cases of multiple myelomatosis with very high plasma protein concentrations, particularly plasma globulins, and correspondingly very high plasma viscosity values.

It can be seen from Figs. 2, 3, and 4 that gum acacia, polyvinyl pyrrolidone, and dextran greatly influence the sedimentation rate. In each solution, as the concentration increases, the maximum erythrocyte sedimentation rate also increases, until a peak is reached. From this point, as the concentration and, hence, viscosity of the solution increase, so the maximum erythrocyte sedimentation rate decreases to approach the normal rates for erythrocyte suspended in plasma (0.08 mm./min., Rourke and Ernstene, 1930).

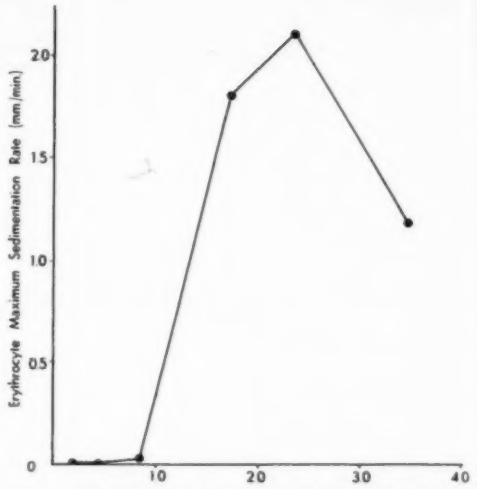

FIG. 3.—The maximum sedimentation rate of red cells suspended in various dilutions of polyvinyl pyrrolidone at a packed cell volume of 45%.

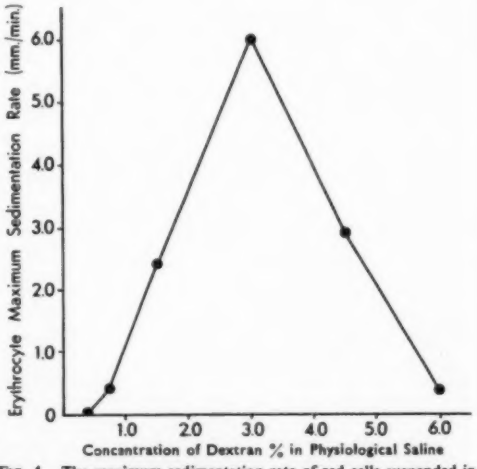

FIG. 4.—The maximum sedimentation rate of red cells suspended in various dilutions of dextran at a packed cell volume of 45%.

Discussion

It is suggested that the three blood samples in Fig. 1 which lie outside the rough line formed by

the other 96 specimens are in fact specimens in which the plasma viscosity is so great that, although red cell aggregation is intense (confirmed by examination of wet films), the fall of the aggregate is impeded by the viscosity of the fluid.

That this is possible may be seen in Figs. 2, 3, and 4. These figures show that as the viscosity of the three solutions increases, so at first does the maximum erythrocyte sedimentation rate until a maximum rate of sedimentation is reached. Beyond this peak, increase in the viscosity of each solution is accompanied by progressive decrease in the maximum rate of sedimentation. Wet films of the red cells in these solutions revealed the presence of intense aggregation, and in the sedimentation tubes the cell columns appeared granular. This granular appearance is common in rapidly sedimenting samples if the columns are viewed in a bright light.

While the erythrocyte sedimentation rate is a useful and easily performed test for changes in the plasma proteins in disease, it is suggested that in some cases in which the plasma viscosity is greatly increased the plasma viscosity is a much better clinical test. Hardwicke and Squire (1952) found a direct correlation between the maximum erythrocyte sedimentation rate and the plasma viscosity, but the highest value for the plasma viscosity ratio relative to water was 2.65. Of the 99 blood samples examined in experiment A, 96 had plasma viscosity ratios ranging between 1.47 and 2.65 and the three outstanding blood samples had plasma viscosity ratios of 3.65, 4.77, and 5.96 respectively.

Thus the plasma proteins and the three other hydrophilic colloids, dextran, acacia, and polyvinyl pyrrolidone, affect the erythrocyte sedimentation rate in two ways; first, by stimulating the formation of large aggregates they increase the sedimentation rate, and secondly, by increasing the plasma viscosity they impede the fall of the aggregates and hence slow the sedimentation rate. It has been shown that either action may overshadow the other, depending on the concentration and viscosity of the colloidal solution. It is suggested that any attempt to correlate the plasma viscosity and the erythrocyte sedimentation rate should allow for this.

Summary

Hydrophilic colloids, such as gum acacia, polyvinyl pyrrolidone, dextran, and plasma proteins, accelerate the erythrocyte sedimentation rate by stimulating red cell aggregation, and also diminish the erythrocyte sedimentation rate by their viscosity slowing the rate of fall of the red cell aggregation.

Which of these two actions of the colloid preponderates is determined by the viscosity value of the solution. As the viscosity of the solution of colloid rises, so the erythrocyte sedimentation rate increases until a point is reached, beyond which any increase in the viscosity of the solution depresses the erythrocyte sedimentation rate.

The significance of these findings is discussed in relation to attempts to relate the erythrocyte sedimentation rate and the plasma viscosity quantitatively.

The specific gravity difference between the red cell and the plasma probably does not exert much effect on the erythrocyte sedimentation rate.

I am grateful to Professor M. Maizels for his encouragement and helpful criticism, and to Dr. J. S. Lawrence for the loan of a viscometer of his design. Also I am indebted to Mr. W. Please, of the Department of Statistics, for the development of the empirical formula.

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ASSOCIATION OF CLINICAL PATHOLOGISTS: 52nd GENERAL MEETING

The fifty-second general meeting of the Association of Clinical Pathologists was held at Harrogate from April 8 to 10, 1954.

For the first session the chairman was Dr. J. V. Wilson.

Myelofibrotic Anaemia in Hodgkin's Disease

J. M. FULLERTON said that, although anaemia had been recognized as a constant feature in Hodgkin's disease, only one recorded case of an aplastic or myelofibrotic type is recorded in the literature.

A male patient was seen showing the features of anaemia, loss of weight, a palpable spleen, and a few enlarged peripheral lymph nodes. Five marrow punctures, including a trephine, suggested the diagnosis of myelofibrotic anaemia or aplastic anaemia. A lymph node biopsy six weeks before death suggested the possibility of a diagnosis of Hodgkin's disease.

Post-mortem findings showed a typical Hodgkin's picture of microscopical involvement of bone marrow of ribs, vertebrae, sternum, iliac crest, and long bones.

The Saline Fragility of Leucocytes

H. G. H. and D. L. RICHARDS (Winchester) described a technique for measuring the saline fragility of leucocytes.

The results are plotted on a graph in the form of two curves, one representing the total cell bodies per c.mm. of blood added to each tube, and the other a curve representing the number of cells still remaining refractile in the solution.

The median leucocyte fragility for 50 normal bloods and a large number of abnormal bloods has been determined. Eighty-four per cent. of normal bloods have median leucocyte fragility between 0.300% and 0.350%. The extreme limits of normal are 0.250% to 0.385%.

The Deficiency Theory of Blast Cell Leukaemias

E. K. BLACKBURN (Sheffield) and L. G. LAJTHA (Oxford) presented evidence against the theory that acute blast cell leukaemia is a deficiency disease.

In more than 60% of over 200 normal bone marrow cultures an absolute increase in the number of polymorphonuclear leucocytes was found during the first 24 hours of the culture. Not infrequently, cultures from cases of chronic myelocytic leukaemia showed a similar increase in the number of polymorphs. On the other hand, no rise in the number of polymorphonuclear leucocytes was observed in 24 series of cultures from 15 patients with acute myeloblastic, paramyeloblastic, or monoblastic leukaemia. Features of non-specific differentiation from blast cells could not be identified with normal maturation. No difference could be detected between blast cells cultured in normal as opposed to acute leukaemic sera.

Study of daily blood counts before and after 52 transfusions in 39 patients with blast cell leukaemia revealed definite improvement in three cases, dubious improvement in seven cases, rapid deterioration in five cases, and probable deterioration in four cases. In the remainder, 33 out of 52 transfusions, there was no significant change.

Frequent or serial fresh plasma transfusions in two cases of acute leukaemia failed to induce significant clinical or laboratory improvement.

For the second session on the opening day Dr. W. H. McMenemey was in the chair.

The Serum Antithrombin Test in Fibrocystic Disease of the Pancreas

J. C. W. MACFARLANE said that the use of the antithrombin content of plasma as an aid to laboratory confirmation of the clinical diagnosis of fibrocystic disease of the pancreas was originally suggested by Innerfield, Angrist, and Benjamin (1951). With a slightly modified technique and using serum in place of plasma, this test has been used at The Hospital for Sick Children, Great Ormond Street, since December, 1951. The technique employed has not altered from that published by MacFarlane (1952).

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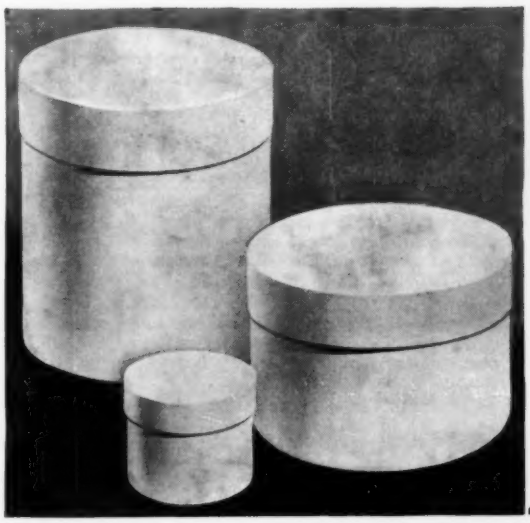

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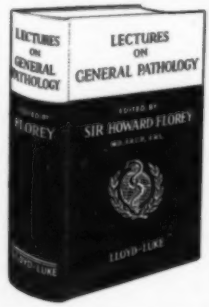

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LECTURES ON GENERAL PATHOLOGY

EDITED BY

SIR HOWARD FLOREY

M.D., F.R.C.P., F.R.S.

¶ THIS book is based on the lectures given to medical students at the Sir William Dunn School of Pathology, Oxford. It is designed to give a background to modern work and to indicate some of the conclusions that have been reached by use of experimental methods in the study of disease. Special attention is given to the effect of various kinds of injury. For example, the course of inflammatory and immune reactions to infection are dealt with at length, and the special characteristics of injury due to trauma and to radiation are also considered.

¶ Although the lectures are designed for the medical undergraduate, it is thought that they may be of value to postgraduate students and to practising physicians and surgeons, as some of them give an insight into modern methods of research and reflect the ideas current among research workers.

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The test has been used in 102 cases of fibrocystic disease of the pancreas, confirmed by examination of the duodenal juice, and in 92 of them, or 90%, the serum antithrombin content has been found to be considerably lower than that found in normal serum. The results have ranged from 18% to 68% of normal. Some 750 "normal" sera have been examined, the cases being of the same age group, including 114 cases of recurrent chest infections, 36 cases of coeliac disease, 45 cases of "failure to thrive," 27 cases of fatty diarrhoea, and 73 relatives of 22 proven cases. There have been six "false positive" results; one a case of eczema, one of advanced miliary tuberculosis, and four showing chronic respiratory infections.

No rationale for the test can be offered, but it is thought to offer a simple and reliable, if empirical, aid to diagnosis.

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Risks from Chronic Irradiation and their Haematological Control

R. H. MOLE said that in human cases of serious anaemia due to radiation little if any warning was given by the white cell count preceding the illness. He discussed the evidence, both clinical and experimental, and it was concluded that it was not likely that changes in the blood count would be observed unless exposure to radiation was many times the maximum permissible. Damage to the gonads occurred with smaller dose rates of radiation. Physical measures of control were considered preferable to haematological methods.

[We hope to publish this paper in full in a future issue of the journal.—ED.]

Observations on Temperature Levels in Hot Air Ovens

R. BARRINGTON BROCK and E. M. DARMADY (Portsmouth) described how multipoint thermocouples were used to test ovens loaded with syringes. Observations were made on two gas, three electric, and two electric ovens with fans. The experiment showed the variations over 40° C.–50° C. in the gas oven and that in many sites the temperature was considerably lower than that of the thermometer reading. In the electric oven the final variation of temperature varied from 28° C. to 12° C., and the take-up of heat was slow and variable. In the electric ovens fitted with fans the final variation of temperature was less than 10° C., and the take-up of heat was immediate and reliable. Tightly packing the syringes prevented the absorption of heat in whatever oven was used. The loading of syringes while the oven was hot made no difference to the amount of heat absorbed provided the temperature was allowed to regain the selected degree. In the ovens fitted with fans heat was probably taken up more quickly and more evenly. It was suggested that heat treatment in these ovens need not be so long as in the electric oven but that the gas oven could not be regarded as reliable. A plea was made for the description of ovens when loaded rather than the normal practice of describing when empty, since, apart from one gas and one electric, all the ovens investigated showed variation when empty of less than $\pm 3°$ C. The tight packing of syringes was condemned. Preliminary experiments with autoclaves showed similar discrepancies.

On April 9 the chairman at the first session was Professor R. A. Willis and at the second Dr. R. W. Fairbrother.

In the afternoon Professor C. H. Gray took the chair.

During the afternoon on April 9 demonstrations were shown at the Royal Bath Hospital, Harrogate.

Pneumocystis Carinii in Interstitial Plasmacellular Pneumonia

H. S. BAAR described the first case of interstitial plasmacellular pneumonia outside the European continent. The finding of characteristic honeycombed masses in air-spaces, which were identified by Vaněk and Jirovec as the protozoon *Pneumocystis carinii*, was confirmed. Histochemical reactions of the honeycombed masses were reported. The case was associated with cytomegalic inclusions of salivary glands and the significance of this association was discussed.

Nephrocalcinosis and Hypercalcaemia in an Infant

I. M. P. DAWSON and W. S. CRAIG (Leeds) described a boy aged 5 months who presented with anorexia, vomiting, constipation, and hypotonia, persisting, with periods of irregular fever, till death at 11 months. Investigations showed raised serum calcium, blood cholesterol, and urea nitrogen levels. Other biochemical findings, including plasma bicarbonate, alkaline phosphatase and chlorides, were normal. Pus cells were found in the urine which was acid. Radiographs showed increased density of the skull and long bones. Necropsy revealed bronchopneumonia, without parathyroid hyperplasia or gross changes in other organs.

Microscopically, the lungs showed unresolved pneumonia. In the kidneys there was patchy hyaline change in some glomeruli, and both calcium-containing and eosinophilic casts in distal and collecting tubules, with some calcium deposition in surrounding tissue. The proximal tubules showed vacuolation and degeneration with calcium deposits in degenerate areas. The case was considered similar to those described by Lightwood (1952) as idiopathic hypercalcaemia. The pathological changes were those of a nephron nephrosis of unknown aetiology, and the biochemical disturbances probably secondary to primary renal damage.

REFERENCE

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The Pathology of Ulcerative Colitis

CUTHBERT E. DUKES said that the modern surgical treatment of ulcerative colitis by colectomy had provided a new opportunity for the study of the lesions in severe cases. These always include widespread inflammation of the mucosa, often accompanied by extensive superficial ulceration, congestion of the submucosa, the presence of inflammatory polyps and strictures.

A patient with severe ulcerative colitis is threatened with many dangers such as haemorrhage, toxaemia, and perforation. There is also a less obvious yet persistent menace increasing with time, namely, cancer. When cancer follows chronic ulcerative colitis the malignant tumours are frequently multiple and atypical in appearance, grow rapidly and metastasize early.

Almost all who during recent years have enquired into the relationship between ulcerative colitis and carcinoma have reached the conclusion that colitis predisposes to cancer and that the extent of this predisposition has been underestimated in the past because of difficulties in diagnosis, both clinical and pathological.

Dr. Dukes explained that his experience was based chiefly on the examination of colons removed by colectomy from severe cases of ulcerative colitis. In a consecutive series of 120 cases of severe colitis carcinoma was found seven times, an incidence of 5.8%. The average age of these patients was 42 years. The average duration of symptoms of ulcerative colitis before the onset of cancer in this series of cases was 15 years.

If the question is asked, "How often does cancer follow ulcerative colitis?", the answer must obviously depend on the severity of the disease and on the duration of symptoms of the cases being considered. Such a question can only be answered in general terms by saying that any patient in whom severe ulcerative colitis has lasted more than 10 to 15 years has entered a phase of life when intestinal cancer has become a serious risk.

Some Observations on the Pathology of Sjögren's Syndrome

B. S. CARDELL reported the necropsy findings in three cases of Sjögren's syndrome and discussed them in relation to four cases recorded in the literature. There appeared to be two main components, (1) atrophy of certain glands and dryness of their associated mucosae and (2) rheumatoid arthritis. In the first component the essential lesion was in the conjunctival, lacrimal, and salivary glands and the submucous glands of the respiratory and upper alimentary tract. The distribution of the glandular lesion in the seven necropsy cases was lacrimal and salivary, seven, conjunctivae, six, mouth, tongue, and larynx, five, oesophagus, four, nose and trachea, three, and bronchi, two. In the earlier stage there was a predominantly lymphocytic cellular infiltration producing glandular enlargement and parenchymal atrophy which resulted in impaired function. Eventually both parenchyma and the cellular infiltrate disappeared and were replaced by fibro-adipose tissue. Dryness of the associated mucosae was a result of the glandular atrophy.

The nature of the underlying pathological process remained obscure. It was possible that the glandular atrophy and the rheumatoid arthritis were both manifestations of collagen disease. This theory received some support from the observation that cortisone produced symptomatic improvement in these cases and from the occurrence of polyarteritis nodosa in two of the seven necropsy cases.

An Incidence of Miliary Tubercles in Bone Marrow Related to Diagnostic Needle Biopsy

J. L. EMERY and N. M. GIBBS (Sheffield) said that the clinical diagnosis of miliary tuberculosis using the present methods of radiological appearance of the lungs and examination of the eye for choroidal tubercles was known to reveal the disease in a little less than half of the cases.

A systematic study of 0.1 ml. of bone marrow from 44 children who had died with generalized miliary tuberculosis revealed miliary tubercles in 13 cases.

In these 44 children a clinical diagnosis of miliary tuberculosis had been made by radiography of the chest in 13 and examination of the eye in 14 cases. These two methods in conjunction produced the diagnosis in 18 cases. Marrow biopsy would theoretically have enabled the clinical diagnosis to be made in a further five cases.

It was suggested that bone marrow aspiration biopsy had a place in the diagnosis of generalized miliary tuberculosis.

Cristobalite Granuloma

T. C. MORTON said that cristobalite was a rare crystalline form of silica occurring in nature. Quartz and amorphous silica are converted into cristobalite by heating them above 800° C.

The case reported was that of a single woman of 44 who had a simple appendicectomy carried out in 1940. Thirteen years later a lumbar sympathectomy was carried out retroperitoneally on the right side and a small lymph gland found incidentally was sent down for biopsy. This was at first suspected to be the site of a hyperplastic tubercular lymphadenitis. Owing to the success of the operation a left lumbar sympathectomy was carried out through an abdominal incision and a chain of grossly enlarged para-aortic lymph glands was found on the left side. There were no peritoneal adhesions or the usual talc granulomatous plaques or nodules. The lymph glands were removed and showed a marked foreign body reaction with numerous inclusion bodies visible in ordinary stained sections, especially very prominently under Nicoll's prisms. A re-examination of the original biopsy from the right side under Nicoll's prisms showed identically similar particles. Professor King and Dr. Nagelschmidt showed that these inclusion bodies were cristobalite in addition to talc.

It is known that the talc dusting powder in use in 1940 was of inferior quality and it is surmised that quartz was present as a contaminant together with cristobalite. The interesting feature was the wide dispersion from the site of the operation and the fact

that the proliferative foreign body reaction was confined solely to the lymph glands.

M. J. H. SMITH and M. E. H. PESSELL reported experiments and results of "The Estimation of Serum Acid and Alkaline Phosphatase Activity with 4-Amino-antipyrine." This paper will be published in the August issue of the journal.

A Calcium Tolerance Test

B. E. C. NORDIN (Postgraduate Medical School of London) described an intravenous calcium tolerance test which might assist in the diagnosis of certain types of metabolic bone disease. Calcium gluconate (15 mg. calcium per kg. body weight) was given intravenously in normal saline over a four-hour period after the subject had been on a low calcium diet (100 mg.) for three days. In nine normal subjects the mean urinary calcium excretion during the 12 hours following the start of the infusion was 50.7% of the administered dose: two standard deviations either side of the mean gave a normal range of 38 to 62%. In seven cases of proven osteomalacia the excretion was always less than 25%. In nine cases of osteoporosis the excretion was well above the normal range in three (all young adults) but within the normal range in six (all post-menopausal osteoporosis). An interesting aspect of the test was the fall in urinary phosphate clearance which followed the infusion and which was most marked in the osteomalacic group. It seemed possible that this was due to suppression of activity in the parathyroid gland.

Paper Chromatography of Urinary 17-Ketosteroids

SHEILA MCDONOUGH and ARTHUR JORDAN said that a considerable number of neutral 17-ketosteroids had been found in urine, but the methods hitherto employed for their separation had not been suitable for routine use. Chromatography using impregnated filter paper can give excellent results but in our hands proved to be very uncertain, but satisfactory separations using a cyclohexane when a 20% (v/v) methanol system on Whatman No. 31 paper and working at a temperature of 34.8° C. had been found satisfactory. The amount of 17-ketosteroid on the paper must not exceed 400 μg. From this amount, an extract from a normal male gives five or six spots. Work is in progress to identify these spots. The least mobile spot is not yet identified. The next appears to be aetiocholan-3α:11β-diol-17 one, the next aetiocholan-3α-ol-11:17-dione. The fourth spot is not yet identified, it may be androstan-3α:11β-diol-17-one; the fifth spot is not always present, it consists of androst-5-en-3β-ol-17 one. The sixth, and most mobile, spot contains three components: androsterone (androstan-3α-ol-17-one), aetiocholan-3α-ol-17-one, and 3βchloro-androst-5-en-17-one.

Haemoglobin S and Haemoglobin C—A Case with Sickle Cell Haemoglobin C Disease

H. LEHMANN and G. M. EDINGTON said that six human haemoglobins were now known of which five seemed to be alleles genetically: adult (A), sickle cell (S), C, D, and E. Foetal haemoglobin (D) is not usually found after the first year of life, except in thalassaemia and in sickle cell anaemia and in some other anaemias, particularly those which started before the physiological haemoglobin F formation in the infant came to an end. Haemoglobin F is characterized by its resistance to alkali. A, S, and C haemoglobins differ in their behaviour on paper electrophoresis, and, in addition, a feature of S is its tendency to gel at reduced O_2 tension. At *p*H 8.6 A migrates fastest, S less so, and C is slower than both A and S. Various chemical differences have been alleged to exist between A and S: the number of SH groups, or the number of "active" SH groups, or the number of valine molecules are said to be different. The only definite chemical differentiation is shown by electrophoresis at *p*H 11 and 4 (Scheinberg). As the two haemoglobins migrate differently at *p*H 11, the difference cannot be due to the NH_2 groups which are inactive at this *p*H. They do, however, migrate at equal speeds at *p*H 4, suggesting that the different migrating rates at the other *p*H ranges are due to carbonyl groups. D has the same electrophoretic properties as S but does not gel at reduced O_2 tension, E migrates at alkaline *p*H at the same rate as A but differs from A at acid *p*H. D and E have so far been found in one family each, but S and C seem to be widely distributed and their incidence pattern is of anthropological interest. Much work has been done on S in that respect but little so far on C. The present case is the first to be reported from outside the U.S.A. A survey recently carried out jointly with Dr. D. F. Roberts in Southern Sudanese Nilotes, including 75 unrelated Dinkas, failed to show C in that group, a population which also does not have the S haemoglobin. A systematic survey of C incidence in West Africa is now in progress and plans are made to extend this to other parts of Africa.

S can give rise to sickle cell anaemia and it is important to differentiate between the haemolytic anaemia and the sickle cell disease, the condition in which crises may lead to death. The anaemia is similar to that of acholuric jaundice and to the newly discovered "target cell anaemia" associated with C, and to thalassaemia. Similarly as in acholuric jaundice, but to a lesser extent, it is now believed that the abnormally shaped cells are eliminated by the reticulo-endothelial system. The crisis is peculiar to the S-carrying red cell and it is due to sickling of cells *in vivo*. A most important factor in their causation are infections.

The therapy of the homozygous, giving rise to sickle cell anaemia, and the heterozygous, giving rise to harmless sickle cell trait, will have to be modified, and while it will always be true that a very severe case of sickle cell anaemia will be a homozygote and a perfectly healthy S individual a heterozygote, there is a wide scale between the two. Sickle cell trait is not always harmless and in the U.S.A. a disease involving haematuria is now found more and more exclusively in S heterozygotes. There must be factors (modifiers) of which we know as yet little which determine the percentage of haemoglobin

from each gene. In A–S heterozygotes A has up to now always been found to be >S: among S–C heterozygotes none has been so far found in whom the relationship was not C<S.

One difficulty is the discrepancy between observed incidence of sickle cell anaemia and the calculated incidence of S homozygotes. Evidence has recently been brought forward to suggest the protection of S homozygotes against malaria, and the question arises whether malaria protects homozygotes from sickle cell crises. If this could be proven it would go far in helping us to understand the increased finding of sickle cell anaemia in areas where malaria is being eradicated (U.S.A., Greece, certain parts of Africa). Better diagnostic facilities and more intensive search for the condition will not serve as a complete explanation. Some relationship between malaria and sickle cell anaemia would serve as an additional explanation of the survival at high frequency of the sickling gene in areas where malaria is endemic. Sickle cell crises have a sufficiently serious prognosis to justify an examination of the possible therapeutic value of malaria.

Protein and Protein-bound Polysaccharide Abnormalities in the Diagnosis of Amyloid and Allied Disorders by Paper Electrophoresis

I. C. GILLILAND and E. STANTON said that diabetic nephropathy described by Kimmelstiel and Wilson (1936) shows deposits of hyaline-like material in the arterioles and in the glomerulus. MacManus (1950), by periodic-acid-Schiff staining, showed it to be a polysaccharide-containing protein. He suggested that they might be deposited from the blood.

Diabetic retinopathy shows similar changes of polysaccharides in the hyaline arterioles and microaneurysms. Duguid and Anderson (1952) suggested that these also are deposited from the blood.

Initial observations required elaborate chemical estimations (Gilliland, Hanno, and Strudwick, 1954), but it was now proposed to show that the same information could be obtained by a simple method suitable for routine use.

Total proteins and total polysaccharides were measured in the serum and further characterized by electrophoresis on paper. Duplicate papers were run for each sample. Naphthalene black stain demonstrated the proteins and periodic-acid-Schiff stain demonstrated the polysaccharides. The colour density was read with the recording densitometer of Laurence (1954), the Gaussian curves completed, and the relative areas measured by planimetry.

No evidence of abnormal polysaccharide was found in a series of cases of diabetes, but a progressive rise in the total protein-bound polysaccharides occurred as the complications of diabetes increased. The major component affected was the α_2 globulin. The albumin fell as α_2 rose, so that four groups could be well differentiated by comparing the albumin α_2 ratio without the need for chemical estimation of the total. As the polysaccharides are protein-bound an indication can be derived from examination of the protein pattern alone. These protein changes are consistent and of diagnostic value in diabetes with Kimmelstiel-Wilson complications.

A useful index of complications could be obtained from simple electrophoresis on paper of proteins and estimation on the albumin/α_2 ratio so derived. This index was tested in a new series of diabetic patients suffering from three grades of diabetic retinopathy and the index correlated with the severity of the complications.

It had been shown by many people (Gutman, 1948; Marrack and Hoch, 1949; Lever, 1951) that a rise in protein-bound polysaccharides in the serum is not specific for diabetes but occurs in a wide variety of conditions. Acute rises in protein-bound polysaccharides occur after many feverish or traumatizing episodes, but return to normal. This has been studied particularly in rheumatic fever. Chronic rises more comparable to diabetes occur in rheumatoid arthritis and in untreated tuberculosis amongst other conditions.

If the lesions of the diabetic complications occur as a result of the deposition of these protein-bound polysaccharide materials in the vessel walls and in the kidneys, then similar changes should occur in these other conditions. Both rheumatoid arthritis and tuberculosis may terminate in amyloid disease and the kidney stained by periodic-acid-Schiff stain shows a remarkable similarity to Kimmelstiel-Wilson lesions. The serum of patients with proven amyloid disease examined by the same electrophoretic technique was also similar to those of the diabetics with Kimmelstiel-Wilson lesions.

Estimations of the albumin/α_2 ratio in the serum of patients with uncomplicated rheumatoid arthritis of varying durations showed a steady fall with duration. The albumin/α_2 ratio below 3 was of diagnostic help in amyloid disease.

Seibert *et al.* (1947) showed that the total serum protein-bound polysaccharides are cumulative and that the presence of two diseases such as tuberculosis and diabetes leads to a greater amount being present. The albumin/α_2 ratio in a few patients with tuberculosis and rheumatoid arthritis showed albumin/α_2 ratios diagnostic of amyloid disease which had developed in a period very notably shorter than in the cases with uncomplicated rheumatoid arthritis. In each of these cases the amyloid disease was proved by biopsy.

The albumin/α_2 ratio in several cases of osteoarthritis showed no change.

Sera were examined from four cases of amyloid disease occurring as a result of long-standing tuberculosis and in these the albumin/α_2 ratio was again below 3.

A number of cases of uncomplicated sarcoidosis proved by biopsy were also examined. The albumin/α_2 ratio was lowest in four cases characterized by gross enlargement of liver and spleen. One was shown to have characteristic changes of para-amyloid disease at necropsy.

Our observations in rheumatic fever confirm the rise in protein-bound polysaccharides initially, returning to normal. The albumin/α_2 index can be calculated from the figures given by Jackson *et al.* (1953) on 303 children with rheumatic fever using the Tiselius method. These show a progressive rise in the albumin/α_2 index as the disease progresses.

Thus the albumin/α_2 index gives useful information of prognostic and diagnostic value in a wide variety of disorders.

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The meeting ended on April 10, when the chairman at the first session was Dr. H. R. R. Mavor and at the second Dr. A. G. Signy.

The Isolation of Pasteurella septica from the Sputum in Bronchiectasis

J. M. TALBOT said that *Past. septica* (*syn. multocida*) had now been described on numerous occasions as having been isolated from pathological conditions in man. The majority of examples were from wounds inflicted by animals, but there were a number of cases, most of which were reported from the U.S.A., in which no direct animal contact was known. Prominent in this latter group are those cases in which the organism seems to live as a symbiont in the respiratory tract of those patients with disease of the bronchi, lungs, or nasal sinuses, especially those with bronchiectasis. The persistent occurrence of this organism in the sputum of a patient with bronchiectasis has not previously been reported in this country, but such a case was here described in which the organism was satisfactorily identified by biochemical and pathogenicity tests. There is a strong possibility that more examples of this condition may be forthcoming if a careful watch is kept for them.

The Bacteriology and Pathogenesis of Brain Abscess

L. PARKER reviewed 70 cases of brain abscess investigated at the Manchester Royal Infirmary since January, 1948.

A comprehensive technique was used for the isolation of the organisms. Blood agar plates were incubated aerobically, microaerophilically, and anaerobically. Strict anaerobes were found on 12 occasions and many facultative anaerobes grew better on anaerobic or microaerophilic culture than on aerobic culture when first isolated. Chloral hydrate-blood agar was used when the presence of *Proteus vulgaris* was suspected. Liquid media, particularly a modification of Brewer's thioglycollate medium, were valuable when only a small number of organisms were present.

The organisms most frequently isolated were:

Proteus vulgaris	15
Staph. aureus	13
Non-haemolytic streptococci	11
Bacteroides group	5
Anaerobic streptococci	5

No pneumococci or fungi were isolated.

In 15 cases all cultures were sterile.

The bacterial flora of an abscess was related to the primary focus.

In many of the abscesses due to direct spread of infection the route of spread was easily traced; in some cases there was a bone defect, and in others thrombosed blood vessels were seen.

In abscesses caused by blood-borne infection the route of spread was less obvious. In a few cases there was clinical evidence of bacteraemia before the presence of the abscess was recognized; but the relative importance of bacteraemia and septic embolism (by the systemic arteries or the vertebral veins) in the pathogenesis of haematogenous brain abscesses is difficult to assess.

The Epidemiology of Proteus vulgaris Infections

A. C. CUNLIFFE said that within recent years the importance in surgical infections of Gram-negative bacteria, such as *Pseudomonas pyocyanea* and *Proteus vulgaris*, which are relatively insusceptible to penicillin and the majority of the new antibiotics, had increased. It is generally recognized that a large number of *Ps. pyocyanea* infections are the result of cross-infection in hospital. There is much less knowledge about the epidemiology of *P. vulgaris*.

In the work described the serological classification of *P. vulgaris* had been developed to provide an accurate method of subdividing the species and by this means the epidemiology of *Proteus* infections in a number of populations had been elucidated. The typing of strains isolated at a general hospital and from patients in a burns unit provided evidence suggesting that infection with *Proteus vulgaris* occurs in two ways; (*a*) as the result of self-infection from the gut, and (*b*) by cross-infection.

Evidence for cross-infection was suggested by the finding of the same type of *P. vulgaris* in seven of nine infected burns in one ward of the burns unit. The distribution of types in this and other wards suggested that infection took place in the ward rather than during dressings at the common dressing station. In the general hospital no evidence of cross-infection was found; self-infection was suggested by the fact that in the majority of patients the somatic and flagellar antigens of the strain isolated from the faeces were identical with those of the strain isolated from the site of infection.

A Seasonal Incidence of Toxoplasma Infection?

J. K. A. BEVERLEY and C. P. BEATTIE said that the birth dates of patients born with severe manifestations of congenital toxoplasmosis (hydrocephalus, intracerebral calcification, and chorio-retinitis) showed a peak from April to July; of those born with minor manifestations only, a peak from October to March. If the birth dates of those born with severe manifestations are antedated three months and a histogram made by months from them it will be found to coincide with a histogram made of the birth dates of cases born with minor manifestations. This is in keeping with the supposition that children born of mothers infected during the middle of their pregnancy may show major manifestations and those born of a mother infected late in pregnancy minor manifestations. It also suggests a seasonal incidence of infection in autumn and winter.

Carrier Medium in Pertussis Diagnosis

D:. B. W. LACEY described his carrier medium for isolation of *B. pertussis* and showed the greater ease of isolation using his selection medium.

J. clin. Path. (1954), 7, 175.

A REVIEW OF THE VARIETIES OF HUMAN HAEMOGLOBIN IN HEALTH AND DISEASE

BY

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Haemoglobin is a member of the important class of conjugated proteins, the haem-proteins. The members of this class enter into a wide diversity of reactions in the living organism concerned with oxidation-reduction and oxygen transporting mechanisms. All possess the common structural feature of iron-porphyrin prosthetic groups united with protein; variation in the nature of the haem occurs (cytochrome), but protohaem (ferro- or ferri-protoporphyrin IX) is common (haemoglobin, catalase, horse-radish peroxidase). The nature of the protein and of the linkages to the prosthetic group are of great importance in determining the type of activity of these proteins. In all higher animals, haemoglobin is concerned principally with oxygen-transport, this property being determined largely by the coordination of ferrous iron in the protoporphyrin molecule, and the nature of the linkage of the four haem groups to the surface of the globin.

Haemoglobin is by no means confined to the erythrocytes of higher animals, and is of extremely widespread occurrence in nature (Anson and Mirsky, 1925). For instance, it occurs in the root-nodules of leguminous plants containing rhizobium, and plays a part in nitrogen-fixation (Virtanen and Laine, 1946; Keilin and Smith, 1947). Haemoglobin is also found dissolved in the blood of the crustacean genus *Daphnia*, particularly under conditions of low oxygen tension, and in the partho-

genetic eggs (Fox, Gilchrist, and Phear, 1951). Haemoglobin also occurs in yeasts (Keilin, 1953), the moulds *Neurospora crassa* and *Penicillium notatum* (Keilin and Tissières, 1953), and the protozoa *Paramecium caudatum* and *Tetrahymena pyriformis* (Keilin and Ryley, 1953). Keilin (1953) points out that non-circulating haemoglobins occur in very variable amounts in cells, and are not necessarily actively involved in the respiration, but may arise in the course of active synthesis of haemo-proteins.

It is not surprising that the globin moiety varies in different haemoglobins, the iso-electric point and amino-acid composition varying from species to species (Lemberg and Legge, 1949), and the molecular weight being very high in haemoglobins from some invertebrates (Prosser, 1950). The haem does not vary in structure. Within single species variants of haemoglobin may occur which differ slightly in structure and properties. Until recently the best characterized intra-species variants were the adult and foetal forms of haemoglobin found in many animals and in man. However, the discovery that sickle-cell haemoglobin differs from normal adult human haemoglobin, that it is formed under genetic control, and that its presence is related to the clinical manifestations of sickle-cell disease has stimulated work in this field. Several genetically determined variants of adult human haemoglobin are now recognized, and Pauling and his colleagues have advanced the concept of a "molecular disease" in conditions such as sickle-cell anaemia, where a variation in a normal protein on the molecular level leads to functional disturbance (Pauling, Itano, Singer, and Wells, 1949).

This review will be concerned chiefly with the variants of human haemoglobin that are known to occur in disease. Besides normal adult and foetal haemoglobin and sickle-cell haemoglobin three other variants have been characterized (Table I).

TABLE I
NOMENCLATURE OF HUMAN HAEMOGLOBIN VARIANTS

	Symbol		
	(*Blood*, 8, 386)	(Itano, 1953a, b)	(Singer and Chernoff, 1952)
Normal adult Hb	A	a	N
Normal foetal Hb.	F	f	F
Sickle-cell Hb	S	b	S
Haemoglobin III (second abnormal)	C	c	
Third abnormal Hb	D	d	
Fourth abnormal Hb (Itano *et al.*, 1954)	E		

It is possible that further forms will be discovered. The recognition of these haemoglobins depends on the application of various physico-chemical methods of protein chemistry. The significance of these haemoglobins in relation to clinical syndromes and the nature of their departure from normal haemoglobin structure must therefore be assessed against the general background of haem-proteins and the physical chemistry of proteins. Techniques, such as electrophoresis, solubility measurement, and spectrophotometry, play a recognized part in this work but future developments may involve many other methods of protein investigation.

Quite apart from the structural variants in haemoglobin that depend upon slight differences in the protein moiety, and which are efficient oxygen-carrying pigments as far as is known, several modifications of haemoglobin may occur in which the haem-group is involved and the oxygen-capacity of the blood reduced. These may result entirely from the effects of toxic chemicals (sulphaemoglobinaemia and most instances of Heinz-body anaemia) or from either toxic or genetic factors (the methaemoglobinaemias).

A number of monographs and reviews deal with various aspects of haemoglobin. Wide general treatment is given in the monograph "Hematin Compounds and Bile Pigments" (Lemberg and Legge, 1949) and the review "Chromoproteins" (Drabkin, 1951). Physico-chemical aspects are dealt with in the Barcroft memorial volume, "Haemoglobin" (1949), and in a review by Wyman (1948). The physico-chemical basis for the recognition of abnormal human haemoglobins has been dealt with by one of us (White, 1954) and to avoid duplication some aspects will be condensed as far as possible in the present work. Haurowitz and Hardin (1954) have recently discussed the protein chemistry of haemoglobin and the haemo-proteins. An excellent general review of the abnormal haemoglobins encountered in hereditary anaemias, especially Hb-S, has been given by Harris (1953), who discusses the genetic aspects of the subject in some detail (see also Gajdos, 1953).

THE OXYGEN-CARRYING FUNCTION OF HAEMOGLOBIN

The outstanding function of intracorpuscular haemoglobin is the transport of oxygen from the lungs to the tissues. In addition, it plays an important part in the transport of carbon dioxide from the tissues to the lungs, and in maintaining the hydrogen ion concentration of the blood.

The ability of haemoglobin to transport oxygen depends upon the ability of the weak acid, haemoglobin, to form a loose molecular compound with

oxygen; the resulting oxyhaemoglobin is a slightly stronger acid at physiological *p*H but readily dissociates on reducing the partial pressure of oxygen: $Hb + O_2 \rightleftharpoons HbO_2$. Under favourable conditions, as within the intact erythrocytes, the reversible oxygenation is effected without any significant oxidation to methaemoglobin. If preparations of haemoglobin are exposed to varying partial pressures of oxygen, which are then plotted against the resulting degrees of saturation of haemoglobin with the oxygen, oxygen dissociation curves are obtained. These are based upon the form of a rectangular hyperbola, but a variety of conditions result in the curve assuming a more sigmoid form. This is notably so with intact red cells (Hill, 1935; McCarthy, 1943), and is advantageous under physiological conditions in that the oxygen is more readily given up to tissues at low oxygen tension.

The specific oxygen capacity is the relation of the oxygen to the iron in haemoglobin (Barcroft, 1928). The iron content of haemoglobin was determined by Hüfner (1894) as 0.334%, and the oxygen uptake as 1.34 ml. per g. of haemoglobin; this is equivalent to the binding of one molecule of oxygen per atom of iron. With crystalline haemoglobin, Bernhart and Skeggs (1943) found that the oxygen capacity was slightly less than that expected from the iron-analysis, though the difference is much smaller than that based on Haldane's (1900) original standard of 13.8 g. of haemoglobin per 100 ml. of normal blood, with an oxygen capacity of 18.5 ml. of oxygen/100 ml. King, Gilchrist, Wootton, Donaldson, Sisson, Macfarlane, Jope, O'Brien, Peterson, and Strangeways (1947) find the B.S.I. standard tube (100% Haldane) is equivalent to 14.8 g. haemoglobin/100 ml. of blood when measured by iron analysis, or 14.4 g. when measured by oxygen capacity. The slight difference may be attributable to small amounts of non-functional pigments derived from haemoglobin. The difference is of course proportionately increased when pathological concentrations of methaemoglobin, sulphaemoglobin etc., are present in the blood.

Attempts to analyse the form of the oxygen dissociation curve of haemoglobin were not entirely successful until Adair (1924, 1925a) determined by osmotic pressure measurements the molecular weight of mammalian haemoglobin to be 68,000; this requires four atoms of iron per molecule. Adair (1925b) then considered the process of oxygenation to proceed by step-wise union with the four iron atoms; Pauling (1935) extended this treatment by considering six possible intermediates, with the four haem groups arranged at the corners of a square, and interaction of the haems occurring via the oxygen molecules.

The form of the oxygen dissociation curve of haemoglobin solution and of whole blood is influenced by a number of factors, such as the electrocytes present, temperature, partial pressure of carbon dioxide present (Bock, Field, and Adair, 1924), and hydrogen ion concentration. Increase in the last three factors all produce a more sigmoid curve. The concentration of haemoglobin is important, dilute solutions giving more hyperbolic curves whilst concentrated solutions and intact corpuscles give sigmoid curves. These factors are discussed at length by Barcroft (1928, 1946).

Valtis and Kennedy (1954) have recently brought out a point of considerable clinical interest in connexion with transfusion of stored red cells. They found that the oxygen-dissociation curve of cells which had been stored in acid-citrate-dextrose medium at 4°C. was shifted to the left, this effect being progressive with prolonged storage. Transfusion of blood stored for seven days or more resulted in a similar change in the oxygen dissociation curve of the recipient's blood, the effect lasting for several hours, during which time proportionately less oxygen could be released from the blood to the tissues. The change in the oxygen dissociation curve was considered due to changes in the physical, chemical, and electrolytic relations between stored cells and plasma and not to chemical changes in the haemoglobin (Valtis and Kennedy, 1953).

The form of the oxygen dissociation curve is fundamentally influenced by the type of haemoglobin. Thus, wide differences occur throughout the animal kingdom, particularly with regard to the effect of temperature (see Barcroft, 1928). Foetal and adult forms differ, and this will be discussed subsequently. We are not aware of any detailed studies on the oxygen dissociation curve in human blood where the variants, sickle-cell, C, or D haemoglobins are present. Such studies might be of considerable interest, particularly where foetal haemoglobin is also a concomitant.

A good deal is known about the form of the oxygen dissociation curve of haemoglobin in the presence of chemical derivatives such as carboxyhaemoglobin and methaemoglobin. The shift to the left which occurs must contribute to the degree of tissue anoxia, which, in carbon monoxide poisoning particularly, is out of proportion to the amount of abnormal pigment present.

Haldane and Smith (1897) observed that miners could work with a simple anaemia of less than 50% Hb, but reduction of the oxygen capacity to

the equivalent level by carbon monoxide poisoning often produced collapse. Douglas, Haldane, and Haldane (1912) observed that the dissociation curves for both oxygen and carbon monoxide have the same sigmoid form though the affinity for CO is far greater, but that the curve for oxyhaemoglobin becomes less sigmoid in the presence of carboxyhaemoglobin. Roughton and Darling (1944) showed that the curve moves progressively to the left when the percentage oxygen saturation is plotted against partial pressure of oxygen in the presence of 20, 40, and 60% carboxyhaemoglobin, and that this result would be expected from Pauling's (1935) equation for haem-interaction only if the interaction constants were the same for combination of haemoglobin with oxygen and with CO.

Similar observations have been made by Darling and Roughton (1942) in the case of mixtures of methaemoglobin and ordinary haemoglobin, the shift to the left increasing with increasing percentage of methaemoglobin, and being qualitatively the same as in the presence of carboxyhaemoglobin, though quantitatively less. This effect on the oxygen dissociation curve has been confirmed by Gibson and Harrison (1947) in the familial idiopathic methaemoglobinaemia (see also Baikie and Valtis, 1954). The tendency to tissue anoxia in the case of methaemoglobinaemias is important clinically, and may also obtain in sulphaemoglobinaemias, where cyanosis may be very marked in the presence of only 10 to 15% of the abnormal pigment.

ELECTRONIC STRUCTURE OF HAEMOGLOBIN AND ITS DERIVATIVES

The discussion of the nature of the binding of iron to porphyrin in the haem pigments, the combination of haem with globin to form the conjugated protein haemoglobin, and the manner in which the latter combines reversibly with oxygen and irreversibly with various other compounds to give products of physiological or clinical importance requires a brief note on certain modern valency concepts (Pauling, 1940; Palmer, 1944).

In the general process of bond formation between A and B (which may each be neutral atoms or molecules, ions) the following extreme or pure bond types may be concerned :

$$A^+ \ldots B^- \qquad A{:}B \qquad A \overset{+}{:} \overset{-}{B}$$

(a) (b) (c)

In the ionic bond (a) the bonding force is essentially the Coulomb attraction between charge centres of opposite sign. In the crystalline state ionic bonds result in extended lattices in which the individual ions are arranged in a three-dimensional pattern of minimum potential energy. In appropriate solvents ionic compounds dissociate to form free ions which are stabilized by hydration with solvent molecules and the solute molecule does not exist as such.

In the covalent bond (b) each partner of the bonding process contributes one electron to the electron pair which constitutes the bond. In this bond the electron spins (see below) are paired and the bonded state is energetically more stable (by an amount expressed by the bond energy) than the non-bonded state. The extent to which the electron pair is shared between A and B depends on the relative electronegativities of the bonded atoms etc. When these are dissimilar the sharing is in general unequal and there is some charge distribution across the bond.

In the coordinate bond (c) both electrons required to form the two-electron bond are contributed by one partner A of the bond. The bonding process is therefore accompanied by charge transfer from A to B, giving rise to a marked dipole in the sense shown. The special conditions required for coordinate bond formation are a pair of unshared electrons (lone pair) on the donor and a vacant electron orbital on the acceptor.

In practice (excluding special cases as pure covalently bonded diatomic molecules formed from identical atoms) bonds are not pure ionic or pure covalent but have to be regarded as essentially ionic or essentially covalent. Similarly in the coordinate bond, which plays an important role in complex formation, the charge transfer is not confined to the bond, but may be re-distributed through the whole molecule (cf. hexahydrated Fe^{++}ion, below). All three types of bonding are involved in the chemistry of haemoglobin.

The resonance concept of molecular structure is of importance in the structure of the porphyrins and of the haem-proteins. It is sufficient to state that if more than one arrangement of bonds can be written which satisfies the required spatial arrangement of the atoms in a molecule, then the various structures which can be so written may interact energetically to form a hybrid structure which is more stable than any of the individual structures. The gain in stability so achieved is the resonance energy of the molecule, and individual structures which are themselves energetically improbable may by resonance make significant contributions to the character of the resonance hybrid. Thus ionic structures may contribute to a predomin-

antly covalent structure and vice versa. The stabilization arising from resonance provides an explanation for the complexity of bond type, e.g. the partial ionic character of an otherwise pure covalent bond, or the partial double-bond character of bonds which would be of pure single-bond character in the absence of resonance.

An important experimental criterion of bond type much used in the study of haemoglobin is the magnetic character of the bonding entities and of the resulting compound. In the covalent bond the intrinsic magnetic moments of the bonding electrons (which largely arise from their spins) are paired in opposition, so that there is no resulting magnetic moment. In certain metallic atoms, e.g. Fe, some of the valency electrons exist singly in orbitals capable of holding two paired electrons. The spin of the electrons in these singly occupied orbitals is thus unpaired and gives rise to paramagnetism of the metal atom, which can be estimated theoretically and measured.

The electronic structures of haemoglobin and its derivatives have been discussed in terms of the concepts outlined above in an attempt to provide fundamental explanations for their properties. The structures which have been proposed are largely due to Pauling (1949, cf. Lemberg and Legge, 1949) and are based on the principle of approximate electrical neutrality which states " that in a stable molecule, the electronic structure is such as to associate with each atom the number of electrons that makes the residual electric charge of the atom zero, or, at the most a small fraction of an electronic charge ". A simple example is the hexahydrated ferrous ion $(Fe(OH_2)_6)^{++}$ in which each of the six $Fe–H_2O$ bonds has about one-third covalent, two-thirds ionic character, as required by the relative electronegativities of Fe and O. The overall effect of the coordination bonding process is therefore to transfer $6 \times \frac{1}{3} = 2$ units of negative charge to the iron atom, just sufficient to balance the original charge of $+2$ on Fe^{++}; in the complex the net charge of $+2$ is thus distributable over the 12 H atoms of the complex.

Bond Type in Metal Complexes

In haem (ferroporphyrin) magnetic data indicate four unpaired electrons, corresponding to the four unpaired electrons in the $Fe^{++}3d$ orbitals (Lemberg and Legge, 1949). The bonding is therefore essentially ionic, the two pyrrole H atoms dissociating as protons and being replaced by Fe^{++}; the negative charge left on the porphyrin is equally distributed among the four central N atoms by resonance and the net charge of the haem is therefore zero (apart from possible ionization of side chain carboxyl groups (I).

(I)

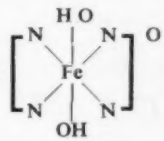
(II)

Haemin and haematin containing ferric iron, Fe^{+++}, resemble haem in having essentially ionic bonds, but the complex has a net charge of $+1$ (II) and is therefore associated with an anion, i.e., ce^-. The exact bond types and the electronic orbitals employed in these complexes is still the subject of discussion.

The electronic configuration of the metal permits the attachment of two more coordinating groups, in which case the stereochemistry of the bonds changes from planar to octahedral. If the additional groups are electrically neutral the net charge of the complex is unaltered (e.g. III) and if not (e.g. OH^-) then it may neutralize the net charge.

(III)

Reduced pyridine haemochrome (dipyridine-ferroporphyrin) N′, N′ are nitrogens of pyridine

(IV)


Haematin (ferriporphyrin hydroxide)

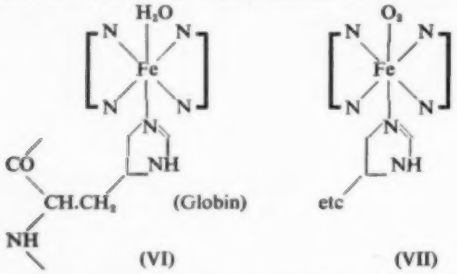
(V)

Pyridine hemichrome (dipyridine ferriporphyrin; parahaematin) N′,N′ are nitrogens of pyridine; X^- is anion, e.g., Cl

The Haem-Globin Bond

The haem-globin bond can be split by acid acetone to give free globin and the appropriate haem and globin, but not by proteolytic enzymes (cf. Haurowitz and Hardin, 1954). The exact nature of the bond is still obscure, but at least one N atom of the globin, contained in a histidine ring, is coordinated to the iron atom. Haurowitz (1949) has presented evidence that the sixth coordination position is occupied by a water molecule; if so, native ferrohaemoglobin is an aquo-complex (VI)

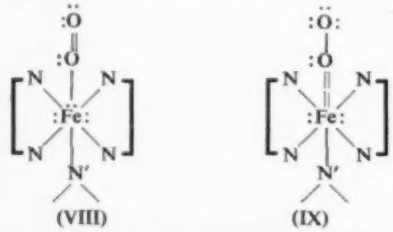
while in oxyferrohaemoglobin the attachment of oxygen requires the sixth position (VII)



(VI) (VII)

Other workers have suggested that two such "haemaffine" groups of the globin are bonded to the iron in ferrohaemoglobin and that one is displaced in oxygen combination. The change in acidity which accompanies the latter, and which most probably involves the NH of a histidine ring, is an important part of the detailed evidence for histidine residues as the bonding agency (cf. Lemberg and Legge, 1949).

In ferrohaemoglobin the paramagnetism indicates ionic bonding, as in the simple haem complexes. Reversible combination with oxygen gives oxyferrohaemoglobin which is diamagnetic, indicating a profound change in electronic structure to the essentially covalent type. The bonding of the oxygen to the metal is in accordance with the principle of approximate electrical neutrality, in which both the metal and the oxygen each contribute two electrons to form what is essentially a double bond, subject, however, to certain resonance processes (VIII, IX).

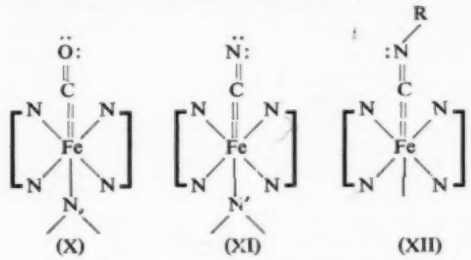


(VIII) (IX)

N^1 denotes N of histidine residue of globin.
(The six electrons around the Fe atom are those which occupy the five 3d orbitals in Fe^{++} and are responsible for its paramagnetism.)

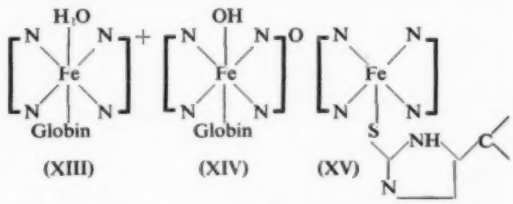
Similar structures can be written for the compounds of ferrohaemoglobin with carbon monoxide, cyanide ion, and the alkyl *iso*cyanides, viz. (X, XI, XII).

Other derivatives of ferrohaemoglobin are discussed by Lemberg and Legge (1949). The nitrosobenzene compound may be of pharmacological importance (Keilin and Hartree, 1943).



(X) (XI) (XII)

The ferrihaemoglobin (methaemoglobin) compounds contain iron in the ferric state (Fe^{+++}), and the resulting net charge is +1. Paramagnetic methaemoglobin with a charge of +1 may be written as (XIII); in alkaline solution the sixth position is occupied by OH^- giving methaemoglobin hydroxide (XIV, alkaline methaemoglobin) with a



(XIII) (XIV) (XV)

fall in magnetic susceptibility. Methaemoglobin derivatives involving combination with fluoride, cyanide (cyanmethaemoglobin), azide, nitric oxide, hydrosulphide, etc., have been described.

The exact nature of the clinically important pigment sulphaemoglobin characterized by an absorption band at about 620 mμ is still obscure (Lemberg and Legge, 1949) and various structures have been proposed, e.g. (XV) involving a covalent S-iminazole band.

Methaemalbumin (ferrihaemalbumin) is an abnormal component of blood plasma in certain diseases associated with excessive haemolysis in which the linkage is probably from the carboxylic acid side-chain groups of the haem to basic groups in the protein, which is serum albumin. The literature on this compound is discussed by Lemberg and Legge (1949); Rosenfeld and Sugenor (1950) have studied the stoichiometry of its formation from serum albumin and ferriprotoporphyrin IX (haemin chloride) and give spectrophotometric data showing the striking 403 mμ Soret band of methaemalbumin which is sharper and nearly 70% more intense than that of the parent haemin at the same molar concentration.

GENERAL PROPERTIES OF HAEMOGLOBIN AS A GLOBULAR PROTEIN

The globin moiety is readily split from haemoglobin with mineral acids in organic solvents, such as hydrochloric acid-acetone (Schulz, 1898). The white precipitate of denatured globin obtained at room temperature is water soluble, but precipitated by ammonia. This globin has often been classified with the histones. However, the amino-acid composition is quite different, and the highly individual character of globin has emerged in work using undenatured globin split from haemoglobin at low temperatures in acid-acetone (Hill and Holden, 1926). Anson and Mirsky (1930) showed that the globin and haematin split at −15°C were capable of recombination, and Jope, Jope, and O'Brien (1949) succeeded in splitting human haemoglobin and recombining it to yield crystalline methaemoglobin, from which crystalline derivatives such as reduced oxy- and carboxyhaemoglobin were prepared, all of which closely resembled their normal counterparts as judged by searching spectrophotometric tests.

The ferrous iron atoms of haemoglobin form a Werner coordination complex with the porphyrin ring nitrogens and with the protein (Haurowitz and Waelsch, 1929), and further evidence for this has been reviewed by Pauling (1949), Lemberg and Legge (1949), and by Wyman (1949). The mutual interaction of haem, protein, and oxygen is illustrated by the decreased affinity of haemoglobin for oxygen in the presence of carbon dioxide (Bohr, Hasselbalch, and Krogh (1904) and the converse mechanism (Christiansen, Douglas and Haldane, 1914) due to the increased acidity of oxyhaemoglobin (Roughton, 1944). Conant (1933) considered that the haems might be bound to two "haemaffine" groups, probably imidazole groups, and the more recent electronic treatment follows the same lines, though a molecule of water may occupy one coordination position in reduced haemoglobin according to Haurowitz (1949).

The relative ease of splitting of the bonds between haem and globin forms the basis of some methods for identifying haemoglobin variants, and will be discussed below.

The Properties of Haemoglobin in Solution

The Molecular Weight.—Unlike invertebrate haemoglobins, which vary widely in molecular size, vertebrate haemoglobins all have a molecular weight of 68,000 and the molecule contains four haem groups. Adair (1924, 1925 a and b) established this value by osmotic pressure measurements on salt-free, 4% solutions at −0.6° C, and this was confirmed by subsequent measurements of the diffusion constant (Northrop and Anson, 1929), and of the sedimentation constant in the ultra-centrifuge (Svedberg and Nichols, 1927).

Burk and Greenberg (1930) found that in concentrated urea solution (6.66 M) between *p*H 7.3 and 9 the molecular weight of haemoglobin is halved, as judged by the osmotic pressure. Wyman (1949) found that two haem groups are associated with each half-molecule, and the x-ray diffraction data of Perutz (1949) indicates a diad axis of symmetry passing through the centre of the intact haemoglobin molecule. In the special case of one crystalline form of sickle-cell haemoglobin, the evidence from x-ray diffraction indicates that the molecules in the large unit cell are twice the normal molecular weight.

The iso-electric points of mammalian haemoglobins are usually just on the acid side of 7, whereas for invertebrate haemoglobins the points are usually lower. Slight variation in the iso-electric points of the various human haemoglobins provides the basis for their recognition by electrophoretic analysis (vide infra).

Electrophoresis of Haemoglobin.—The application of "free" or gravitationally stabilized moving-boundary electrophoresis to the analytical and preparative separation of mixtures of proteins in solution is due largely to Tiselius (1937, 1940), who introduced the experimental features which are responsible for the great practical value of the technique. It has been frequently described and critical reviews of its application to the study of plasma proteins (Edsall, 1947), and for preparative separations generally (Svensson, 1948) give adequate literature references (see also Longsworth, 1942). The electric mobility of a globular protein over a wide range of *p*H values is one of the most important methods of characterizing a protein quantitatively and demonstrating its purity.

The first systematic application of boundary electrophoresis to haemoglobins is attributed to Stern, Reiner, and Silber (1945), who cite some earlier work. Stern *et al.* obtained well-resolved single peaks for the haemoglobins (run as HbO_2) from haemolysates of man, dog, and chicken bloods, and minor peaks due to colourless proteins, one of which appeared to be due to membrane or stromal protein. Chicken haemoglobin gave a complex peak, but this possible indication of heterogeneity was not further studied.

The electrophoretic analysis of bloods containing Hb-F, Hb-S, and other abnormal haemoglobins is discussed below.

In free electrophoresis the movement and separation of boundaries between overlapping components of different mobility is observed, usually optically. In zone electrophoresis the components migrate as separate zones and are stabilized against convection by the supporting medium. For low-molecular substances this may be a gel but for work with proteins filter-paper has received most attention. Because of the simplicity of the equipment, the possibility of essentially complete separation of the components of complex mixtures and the small samples required, zone electrophoresis on filter-paper has been used by a large number of workers for the study of proteins, notably the blood serum proteins. General reviews of the subject have been published by Lederer (1951), Tiselius and Flodin (1953), Fisher (1953b), and Martin and Franglen (1954). As pointed out by Tiselius and Flodin (1953), the many types of apparatus that have been described for zone electrophoresis on filter paper all derive essentially from those of Durrum (1950) with a freely-hanging non-horizontal strip; Wieland (1948) with freely-hanging horizontal paper; and Cremer and Tiselius (1950) with supported horizontal paper. Martin and Franglen (1954) have most recently summarized the growing literature on the subject with respect to apparatus and applications. The chief disadvantage of zone electrophoresis, as compared with the boundary method, is its present inability to provide absolute mobility data and iso-electric point estimates because of complicating factors introduced by the supporting medium. These limitations have been discussed by Kunkel and Tiselius (1951), who propose some correction procedures. McDonald, Urbin, and Williamson (1951) have also studied the determination of isoelectric points.

It should perhaps be pointed out that the *quantitative* evaluation of protein zone-electrophoresis patterns on filter-paper is not always easy and has been the subject of much debate (cf. Martin and Franglen, 1954). Quantitative comparisons between free boundary and zone electrophoretic analysis have been made by several workers (Kunkel and Tiselius, 1951; Flynn and de Mayo, 1951; Ott, Huber, and Körver, 1952) and a considerable measure of agreement is generally observed in favourable cases.

Denaturation of Haemoglobin.—A large proportion of the extensive literature on the denaturation of proteins is concerned with haemoglobin, because its characteristic property of reversible combination with oxygen is lost when the globin moiety is denatured and, furthermore, the coordination of denatured globin with haem gives rise to a group of "haemochrome" derivatives with very characteristic spectra.

In relation to the study of abnormal haemoglobins in hereditary anaemias, the most important denaturation reaction that has been used up to the present time is the conversion of HbO_2 into alkaline haematin by alkali at *p*H values in the region of 11.5 to 13. Körber (1866) noted that human foetal HbO_2 was much more resistant to alkali than adult HbO_2 and Krüger (1888, 1925) utilized this same reaction to reveal differences between the haemoglobins of various species. Subsequent work along the same lines has been summarized by Haurowitz and Hardin (1954), and the more recent application to the study of abnormal human haemoglobin mixtures is discussed below. Havinga and Itano (1953) have noted differences in the rate of splitting of globin from Hb-S and Hb-A respectively but no analytical use has yet been made of this finding. Other denaturation reactions that have been studied in the case of Hb-A/Hb-F mixtures are briefly noted below.

The fact that SH groups are either unblocked or formed in denaturation is of interest in the light of Ingbar and Kass's (1951) observation that Hb-S contains one more titratable SH group per mole of haemoglobin than Hb-A; this difference is not retained in 20% methanol in which some degree of denaturation seems to occur.

Amino-acid Composition of Globin

Vertebrate globins are remarkable in containing a very high percentage of histidine (7.8–8.6%; Roche, 1936). This contributes to their basic character, and the invertebrate haemoglobins, containing less histidine, are also less basic (Roche, 1936). The imidazole groups are thought to be of fundamental importance in coordination of the haem iron atoms (Wyman, 1948).

Detailed amino-acid analyses are not numerous. Tristram (1949a, b) has critically summarized the data for horse myoglobin and haemoglobin, and considers that the two proteins differ significantly in composition. The numbers of residues are not simple multiples of 4, suggesting variation in the sub-units. Sulphur amino-acids are low and in native globin the sulphydryl groups appear relatively non-reactive, though reacting with some reagents on denaturation (see Haurowitz and Hardin, 1954). Free α-amino groups are numerous (Chibnall, 1942), and the end-group analyses of Porter and Sanger (1948, 1949) reveal five terminal valine residues for adult human haemoglobin and 43 free ϵ-amino-groups of lysine. These data are of importance in considering the x-ray evidence for

folding of the polypeptide chain within the haemoglobin molecule (Perutz, 1949).

The significance of variation in amino-acid composition will be considered further in connexion with foetal and sickle-cell haemoglobins. From the results of their analyses on various mammalian haemoglobins, including adult and foetal forms, Porter and Sanger (1948) attach considerable importance to the parallel between antigenic specificity and the number of valyl end-groups, and hence the configuration of the polypeptide chain.

Immunological Properties of Haemoglobin

Haemoglobin is a poor antigen and its properties in this respect were once the subject of much debate. The non-antigenic properties of the haem moiety and the extent of the species specificity of various haemoglobins have now been established (Marrack, 1938; Landsteiner, 1945; references and reviews). In view of these findings many attempts have been made to utilize the delicacy and specificity of immunological techniques for differentiation of the various types of abnormal haemoglobins encountered in the hereditary anaemias. As noted below the immunological identification of Hb-A and Hb-F has been achieved by many workers, but the antigenic character of Hb-S does not differ very markedly from that of Hb-A. Hb-C and Hb-D do not appear to have been studied.

In this connexion it should be noted that some workers have obtained antisera containing agglutinins to erythrocytes containing Hb-F and Hb-S by using such cells as antigens. In view of the lack of information on possible differences in the membrane and stromal proteins of erythrocytes containing abnormal haemoglobins it seems necessary at this stage of our knowledge of the subject to envisage the possibility that the antigenic response of the various haemoglobins and of the intact erythrocytes carrying them may be different. This possibility is mentioned by Ikin, Lehmann, and Mourant (1953).

The Solubility of Haemoglobin

The solubility of proteins is very sensitive to such factors as the concentration of neutral salts present and the *p*H. According to the concentration of salts present the minimal solubility occurs at a *p*H value more or less close to the iso-electric point of the protein, where the amphoteric molecule exhibits an overall electric neutrality. The effect of electrolytes is particularly related to their ionic strength, which was formulated by Debye (1923) and Debye and Hückel (1923) as half the sum of all the terms obtained by multiplying the molar concentration of each ion by the square of its own valence. A very full treatment of the physical chemistry of protein solubility is given in the monograph by Cohn and Edsall (1943).

In 1925, Cohn showed that the solubility of haemoglobin and other proteins in concentrated ammonium sulphate solutions could be expressed in linear form by plotting log solubility against ionic strength:

$$\log S = \beta' - K'_s (r/2)$$

where S is solubility in g/l, K'_s the salting-out constant, r/2 ionic strength and β' the extrapolated intercept of the linear portion of the curve on the ordinate axis (log S). Haemoglobin solubility in various salt solutions is only linear over a range of high ionic strengths and closely resembles cystine in behaviour (Cohn, 1936). The salting-out constant K'_s is independent of *p*H and temperature for a given salt and protein, whereas β' is markedly influenced and reflects the amphoteric properties of the protein. Green (1931) obtained series of parallel lines when log S was plotted against r/2 for horse HbO_2 and HbCO at varying temperatures, and similarly at varying *p*H; at 0° C. horse HbCO is about 25 times more soluble than at 25° C. at high r/2, but at low salt concentrations solubility increases with temperature.

Green, Cohn, and Blanchard (1935) studied the solubility of human and horse HbCO in concentrated phosphate buffers, and found such marked differences as to distinguish the two proteins completely. Human HbCO was far more soluble, and both β' and K'_s were greater. Jope and O'Brien (1949) have carried out solubility studies of the type described on human foetal and adult haemoglobins. HbCO, crystallized from adult blood, behaved as a single component when increasing amounts of the solid phase were mixed with 2M phosphate at *p*H 6.7. At this *p*H, adult HbO_2, HbCO, and methaemoglobin had rather similar solubilities and obeyed the Cohn equation over the range of r/2, 4 to 6. On the other hand, reduced haemoglobin was far less soluble; in the range r/2, 3.5 to 4.5, solubility was linear. Amorphous HbCO was more soluble than crystalline as found for other species by Roche, Derrien, and Moutte (1941), and higher solubilities were obtained by dissolving up HbCO than by salting out.

Jope and O'Brien (1949) observed solubility differences between reduced Hb and the isomorphous derivatives HbO_2, HbCO, and methaemoglobin with respect to the effect of temperature. Haemoglobin did not vary in solubility with temperature, whereas the latter three had temperature-solubility curves with minima at about 20° C. Dis-

tinction between foetal and adult HbO_2 was also apparent in this respect; the solubility of foetal HbO_2 increased linearly with temperature from 0° to 25° C., resembling the behaviour of many proteins in dilute salt solutions. Foetal reduced Hb was only slightly more soluble than adult, and similarly was unaffected by temperature.

The distinctions established by solubility measurements have recently acquired great practical importance, particularly with regard to the haemoglobin abnormality in sickle-cell anaemia. Roche and Derrien (1953) have discussed the analysis of human haemoglobins by the study of solubility behaviour, with particular reference to fractional salting-out procedures.

The Shape of the Haemoglobin Molecule

Riley and Herbert (1950) have applied low-angle scattering of *x*-rays to concentrated human haemoglobin solutions, and their results suggest that the unhydrated molecules are squat cylinders or spheres, which are close-packed in concentrated solution and surrounded by a single layer of bound-water 3A thick. Dielectric constant studies are consistent with the molecule in solution being ellipsoidal (Wyman, 1948).

Using the method of *x*-ray diffraction by single crystals, Bragg and Perutz and their colleagues have obtained a considerable body of information on the form of the haemoglobin molecules from a number of mammalian species (Bragg and Perutz, 1952a and b; Bragg, 1952, 1953). Ellipsoidal in form, the haemoglobin molecule is about $60 \times 40 \times 40$ A dry, but when hydrated the layer of bound water increases the dimensions to $65–70 \times 55 \times 55$ A and the volume to 115,000 A^3. Determination of the molecular form was achieved by suspending horse methaemoglobin crystals in various strengths of salt solutions. These penetrated into the unit cells but not into the hydrated molecules, and the resulting density changes reduced the intensity of the lower orders of diffraction; the amplitude changes then depend on the form of the molecule.

The *x*-ray diffraction data have also indicated that the polypeptide chains run parallel to the long, or a-axis of the molecule. The chains have been visualized end-on for horse methaemoglobin (Bragg, Howells, and Perutz, 1952), and probably lie in three heavy inner layers and two lighter outer layers. There is evidence that the polypeptide chains are coiled into cylindrical rods possessing the α-helix structure, that they do not run throughout the length of the molecule but are split by the haems into two parts of average length about 25 A, and that the chains turn corners after running straight for about one quarter the length of the molecule (see Perutz, 1952; Crick, 1952; Pauling, 1953).

The haem groups appear to lie on the surface of the haemoglobin molecule, roughly normal to the polypeptide chains and parallel to one another, particularly in reduced haemoglobin. Their strongly absorbing character confers pleochroic properties on the molecules and crystals, plane polarized light being most strongly absorbed when it vibrates with its electric vector parallel with the plane of the flat haem molecules. Perutz (1939, 1952, 1953) has made considerable use of the pleochroism of haemoglobin derivatives in his crystallographic studies. Keilin (1953) has recently reviewed the chemical evidence that the haem groups probably lie in readily accessible positions on the surface of the molecule.

Crystallography of Human Haemoglobin

It has long been known that human haemoglobin and its derivatives could be crystallized, but recent studies have included thorough examination by the polarizing microscope and by *x*-ray diffraction.

Drabkin (1945, 1946) obtained bipyramids of human oxy-haemoglobin and assigned them to the tetragonal class from their optical properties. This has been confirmed by x-ray diffraction studies, the space group being $P4_12_1$ (Perutz, Liquori, and Eirich, 1951; Bragg and Perutz, 1952a).

Jope and O'Brien (1949) have also crystallized adult HbO_2, HbCO, and methaemoglobin under different conditions, and shown them to be isomorphous, and to belong to the orthorhombic system. Reduced haemoglobin yields different crystals, of the monoclinic system. All these findings have also been confirmed by *x*-ray diffraction studies (Perutz and Weisz, 1947; Bragg and Perutz, 1952a).

Human foetal haemoglobin is crystallographically distinct from the adult pigment (Perrier and Jannelli, 1931; Haurowitz, 1935). Jope and O'Brien (1949) found that foetal HbO_2, HbCO, and methaemoglobin were isomorphous, but probably monoclinic. The reduced haemoglobin was also distinct, and Kendrew (1949) confirmed the difference between adult and foetal types by *x*-ray diffraction. Kendrew and Perutz (1948) carried out detailed *x*-ray diffraction analysis on adult and foetal haemoglobins of the sheep. The results indicated considerable structural differences between adult and foetal forms (Bragg and Perutz, 1952a).

FOETAL HAEMOGLOBIN

It will be clear from the foregoing discussion that numerous differences may be found between adult and foetal haemoglobins in a number of species. These differences have attracted particular attention in man, in relation to the occurrence of a foetal-type haemoglobin in post-natal red cells in various pathological states.

The haem moiety of the two forms of pigment is the same, the differences concerning the structure of the globin and perhaps also the haem-globin linkages. To some extent the establishment that adult and foetal haemoglobins are not identical is a general facet of the problem of molecular individuality of globular proteins, but the relative ease of crystallization of these haemoglobins and certain of their unique properties render the experimental approach easier.

The differences between adult and foetal haemoglobins have been discussed by Lecks and Wolman (1950), and the following list is modified from their review: (*a*) Crystallographic differences ; (*b*) solubility differences ; (*c*) differences in amino-acid composition ; (*d*) immunological specificity ; (*e*) greater stability of foetal haemoglobin towards alkali denaturation ; (*f*) rate of spreading in surface films ; (*g*) electrophoretic mobilities ; (*h*) spectrophotometric differences in the ultra-violet region of the spectrum ; (*i*) differences in oxygen dissociation curves ; (*j*) clinical significance of foetal-type haemoglobin. Kendrew (1949) and Zinnser (1952) also note these differences.

Crystallographic Differences

These have already been discussed. In the case of adult and foetal sheep haemoglobins, the results of *x*-ray diffraction studies indicate fundamental molecular differences between the two pigments (Kendrew and Perutz, 1948 ; Bragg and Perutz, 1952a).

Solubility Differences

The careful studies of Jope and O'Brien (1949) reveal the differences between solubility behaviour of adult and foetal forms of the isomorphous HbO_2, HbCO, and methaemoglobin derivatives, and the reduced haemoglobins respectively. The reduced haemoglobins are far less soluble than the isomorphous derivatives for both adult and foetal pigments, but the foetal reduced pigment is more soluble than the adult. Itano (1953c) found that standard quantities of preparations containing foetal haemoglobin from newborn infants or from patients with thalassaemia major were more soluble in strong phosphate buffer than adult haemoglobin. Karvonen (1949) found that the solubility of mixtures of adult and foetal sheep haemoglobins was more than additive, but subject to rather complex physical effects; abrupt reduction in solubility occurred when the protein concentration was increased beyond the onset of crystallization.

The studies of Wyman, Rafferty, and Ingalls (1944) on the solubilities of adult and foetal bovine HbCO in strong phosphate buffers at *p*H 6.8 indicated that the latter protein is more than six times more soluble than the former over the range of ionic strength 4.9 to 5.5.

Differences in Amino-acid Composition

A number of earlier analyses of the amino-acids of various haemoglobins yielded somewhat variable results (see Lemberg and Legge, 1949, for literature), but Vickery (1944) found that the histidine content of crystalline foetal bovine HbCO was significantly lower ($6.43 \pm 0.04\%$) than that of the adult pigment ($6.81 \pm 0.05\%$), as determined by the dichlorobenzenesulphonate method.

Using the 1:2:4-fluorodinitro-benzene reagent for free amino groups in proteins, Porter and Sanger (1948) found five terminal valine residues and 43 free lysine -amino groups in adult human haemoglobin; the foetal pigment contained an average of only 2.6 terminal valine groups, and 47 free lysine ϵ-amino groups. This probably indicates the presence of two valyl end-groups in the foetal molecule, since some adult haemoglobin occurred in even the earliest, 30-week foetal samples examined spectrophotometrically and electrophoretically by Beaven, Hoch, and Holiday (1951).

Van der Linden (1949) applied microbiological assay methods to the analysis of human globins and found significant differences in composition between newborn and children's (6 months—2 years) globins with respect to seven amino-acids, viz, histidine, *iso*-leucine, threonine, methionine, proline, tyrosine, and valine; the greatest differences were found for *iso*-leucine. The same children's bloods compared with adult bloods showed small but significant differences in glutamic acid, methionine, and leucine, pointing to a difference in composition of these two forms of Hb-A.

The Immunological Specificity of Foetal Human Haemoglobin

The antigenic specificity of the globin in haemoglobins from different animals has been studied extensively (Heidelberger and Landsteiner, 1923 ; Hektoen and Schulhof, 1923 ; Hektoen and Boor, 1931), and the difference between human foetal and adult forms was demonstrated clearly by

Darrow, Nowakovsky, and Austin (1940). Porter and Sanger (1948) consider that there is a relationship between the terminal amino-acid residues and immunological specificity.

Schneider and Levin (1950) obtained rabbit antisera giving specific agglutination of sickle-cell anaemia erythrocytes but not of sickle-cell trait erythrocytes. Ikin *et al.* (1953) pointed out that Schneider and Levin may have been using antisera which specifically agglutinated cells containing Hb-F and not Hb-S, as the former appears to be present in relatively small proportions in sickle-cell anaemia whereas it is absent from the sickle-cell trait condition.

Goodman and Campbell (1953) obtained evidence for antigenic differences between Hb-A and Hb-S using chicken antisera under conditions of maximal precipitation. They concluded that these two haemoglobins both possess a large number of common antigenic determinant groups, but that each carries a small number of such groups that are unique for each type of haemoglobin.

Relatively large differences in specificity were found to exist between Hb-A and Hb-F indicating that only a few antigenic determinant groups are common to this pair of haemoglobins. The immunological behaviour of the minor haemoglobin of sickle-cell anaemia blood was shown to be very similar, if not identical with that of Hb-F, in agreement with the indications that this component is in part at least Hb-F.

Chernoff (1953a) obtained rabbit antisera to haemoglobin solutions prepared from normal adult, cord blood, and sickle-cell anaemia erythrocytes. An anti-Hb-F serum was also prepared using Hb-F freed from Hb-A by partial alkaline denaturation, and appeared to be specific for Hb-F. No such specificity was found between the anti-Hb-A and anti-sickle-cell anaemia sera. This lack of specificity was suggested to be due to the presence of Hb-F in varying amounts in all the antigens used and hence of Hb-F precipitins in all the antisera. In a second paper (1953b) the immunological detection of Hb-F by means of a specific anti-Hb-F serum was put on a quantitative basis and used to detect traces of (up to 1%) Hb-F in normal adult bloods. Comparable or larger proportions of Hb-F were similarly detected in bloods from a large number of haematological conditions and confirmed by parallel estimations of alkali-resistant haemoglobin. Chernoff concluded that the minor haemoglobin constituent observed in these conditions was indeed Hb-F rather than an abnormal "foetal-like" Hb. It may be noted that these most interesting findings depend on both the immunological and denaturation rate methods of detecting Hb-F being sensitive to this component in proportions of much less than 10%, a level at which other methods for detecting Hb-F are of little value. The unequivocal confirmation of all Chernoff's findings may therefore be difficult.

Ikin *et al.* (1953) obtained rabbit sera containing agglutinins to human cord and newborn red cells by using cord blood erythrocytes as antigen. (Anti-human species agglutinins were removed by absorption, the resulting sera being almost inactive against adult human cells.) Their results show that the specific agglutination reaction with infant erythrocytes becomes negligible after four and a half months, in fair agreement with other estimates of the persistence of Hb-F after birth. In connexion with the various suggestions (Chernoff, 1953a and b ; Goodman and Campbell, 1953) that small proportions of Hb-F are present in normal adults, it is of great interest that Ikin *et al.* obtained weakly positive results with six out of 20 normal adult control bloods.

Alkali-denaturation of Adult and Foetal Haemoglobins

The estimation of Hb-F in haemoglobin mixtures by the alkaline denaturation rate method has been the subject of many variations in technique. All procedures are based on the reasonable assumption that in the presence of excess reagent (OH^-) Hb-A and Hb-F are both transformed independently into alkaline haematin by kinetically first-order reactions so that the overall course is the result of two separate rate curves; the steep initial part of the curve is largely controlled by the fast denaturation of Hb-A and the less steep terminal part by the slow change of Hb-F. Either pure haemoglobin gives a simple rate curve which is linear on a semi-logarithmic plot (% unchanged HbO_2 against time), while a mixture gives a semi-logarithmic plot which may be linear initially and finally with a curved intermediate section of extent depending on the composition of the mixture. Extrapolation of the terminal linear portion of the semi-logarithmic plot back to zero time cuts the composition axis at the Hb-F content of the mixture.

Depending on the analytical method used and the *p*H (which greatly effects the absolute reaction rates) various workers have determined the entire reaction curve, starting as near as possible to zero time and extending to 100% reaction (Brinkman, Wildschut and Wittermans, 1934; Brinkman and Jonxis, 1935, 1936; Jonxis, 1949; Betke, 1951; Beaven and White, 1953, and this review, Fig. 1; Baar and Lloyd 1943). The extrapolation to find the proportion of Hb-F is then independent of the

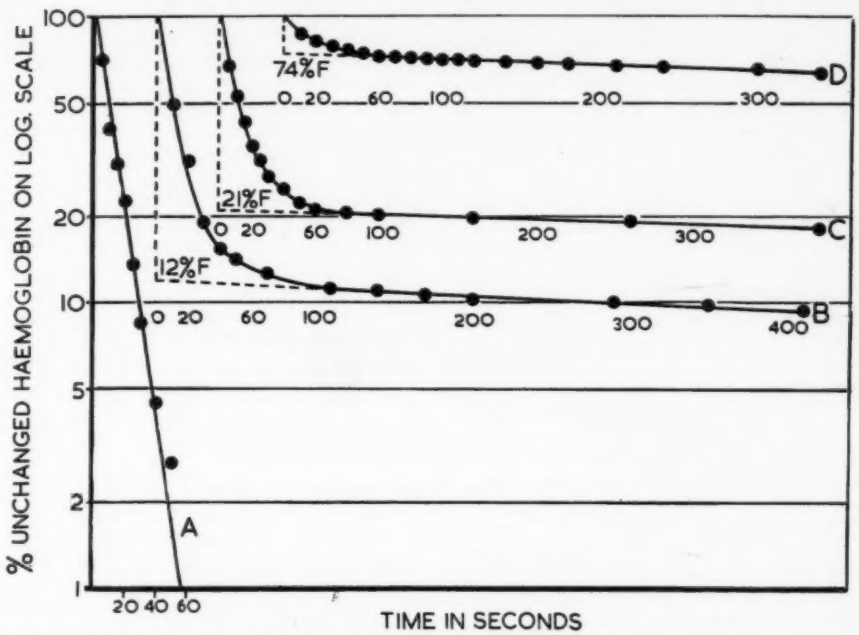


FIG. 1.—Rates of denaturation of adult and foetal haemoglobins in 0.04 N.NaOH at 20° expressed as first-order reaction. A is the sickle-cell trait, and no Hb–F is present. B is Mediterranean anaemia with 12% Hb–F present. C is sickle-cell anaemia in a child of 1 year with 21% Hb–F present. D is cord blood from a normal infant at term with 74% Hb–F present.

absolute rate of the overall reaction and hence of small changes in *p*H and temperature.

Alternatively, the reaction rates for Hb-A and Hb-F differ so markedly (by a factor of about 100) that it is possible to select a reaction period during which only the Hb-A has been completely denatured, so that the residual haemoglobin is all of the foetal type, and is esentially equal to the total Hb-F in the sample. For a *p*H of about 12.7 (N/12 alkali) and temperature of 20°, a period of one minute is convenient (Singer, Chernoff, and Singer 1951a) after which the alkaline haematin is precipitated and the unchanged Hb-F in the supernatant estimated colorimetrically or spectrophotometrically (at 540 mμ) to give a "one minute denaturation value".

In a variant of this procedure Ponder and Levine (1949), working at *p*H 12.15 and 26° C. in an alkali-glycine buffer, precipitated separate replicates of the reaction mixture at periods ranging from 10 to 80 mins. from the start, and by estimating unchanged HbO_2 in the filtrates with a photoelectric colorimeter, obtained five points covering the course of the reaction which could be used to give a linear semi-logarithmic plot (except the 10 min. point). This could be extrapolated to zero time in the usual way to give the Hb-F content of the sample. With this "five-point" method results on artificial mixtures of Hb-A and Hb-F were within 4% of the calculated values and agreed to $\pm 5\%$ with values obtained from the complete reaction curve method used by Baar and Lloyd (1943).

Singer *et al.* (1951b) similarly modified their "one-minute test" to give a "fractional denaturation" curve by the successive sampling of eight to 10 reaction replicates over periods depending on the Hb-F content, estimated by preliminary one-minute tests. These workers pointed out that the fractional denaturation method was relatively inexact and could be used for finding the slope of the denaturation of the alkaline-resistant component (which is directly related to the first order rate constant) only if a sufficiently linear semi-logarithmic plot was obtained. Only about one-third of the sickle-cell anaemia haemoglobins examined by these workers gave denaturation slopes for the alkaline-resistant component comparable with the range of slopes established for authentic Hb-F.

It is unfortunate that the more stable HbCO form of haemoglobin is not suitable for alkaline

denaturation studies, although it has been widely employed in electrophoretic studies. In alkali HbCO is eventually transformed into a haematin-like pigment but the reaction is not susceptible to a simple first-order kinetic treatment (Beaven and White, unpublished work). Methaemoglobin reacts very rapidly at *p*H 12-13 to form alkaline haematin, and a small proportion of methaemoglobin in a haemoglobin sample can often be detected by an abnormally rapid rate of change of absorbance during the first few seconds of the reaction, and by a semi-logarithmic plot which does not extrapolate through the 100% HbO_2 ordinate at zero time. A simple graphical correction can be applied if necessary.

Gardikas, Scott, and Wilkinson (1953) sought other denaturing reagents which might differentiate between Hb-A and Hb-F, and found that in 4.4 M urea foetal methaemoglobin denatured more rapidly than adult methaemoglobin while in sodium salicylate solution the relative stabilities were reversed. An approximate kinetic analysis of their results (Beaven, unpublished) indicates that with urea successive reactions occur, making the method difficult to apply analytically. Betke (1951) was unable to follow the alkaline denaturation of reduced haemoglobin by the simple absorptiometer method applicable to HbO_2. At the present time, therefore, the alkaline denaturation of HbO_2 itself is the most practicable chemical method for the estimation of Hb-F (see also Baar and Hickmans, 1941-42).

It is not essential to use a precipitation method to analyse mixtures of unchanged HbO_2 and alkaline haematin. Their absorption spectra are sufficiently different to permit a direct spectrophotometric analysis using a wavelength of about 630 mμ where the absorbance at equal molar concentrations is optimal for the denaturation product. Alternatively a deep red filter with a pass-band centred around this wavelength is adequate and has been used by many workers in conjunction with commercial photoelectric absorptiometers of conventional design (e.g. Baar and Lloyd, 1943). It is also possible to work at about 540 to 580 mμ or to use a corresponding green filter, in which case the absorbance of the systems falls with the progress of denaturation due to decreasing absorption by HbO_2 (e.g. Larsen, 1951). In our experience, the use of a simple commercial absorptiometer with a deep red filter to measure the complete reaction curve gives excellent linear plots for samples containing no Hb-F. Good terminal plots are obtained when Hb-F is present (Fig. 1) from which the proportion of this component can be found by extrapolation to about $\pm 5\%$. The method is sensitive to about 7% Hb-F with certainty, and the presence of not less than 2 to 5% can be inferred from the "tailing" it produces in the terminal part of the plot. At *p*H 12.8 (N/25 alkali) and room temperatures the half-reaction times ($t_{\frac{1}{2}}$) for Hb-A and Hb-F are about 10 and 1,000 secs. respectively as recorded by earlier workers for this order of *p*H. In view of the lack of sensitivity of the alkaline denaturation method for bloods containing less than about 2 to 5% of Hb-F, and the similar limitations of the ultra-violet spectrographic methods at this Hb-F level, it seems unwise to attach any great significance to reports of traces of Hb-F in various haematological conditions until these findings can be confirmed using analytical methods of increased sensitivity and precision.

Monomolecular Films of Adult and Foetal Haemoglobins

Proteins may be spread on the surface of water or dilute salt solutions to form monomolecular layers, the structure of which can give information on molecular size and form (see Sobotka, 1944). Stable monomolecular films of maximal area form in the neighbourhood of the iso-electric points of proteins, and Brinkman and Jonxis (1935, 1936) have applied the method of Gorter and Grendel (1926) to the study of haemoglobin. The area of spreading on the surface of M/300 phosphate buffers over the range *p*H 6 to 8 was measured, and plotted as area of film per mg. of protein after one minute against *p*H. Foetal and adult haemoglobins differed, the foetal form spreading more slowly than the adult and the maximal area being achieved at *p*H 6.7 instead of 6.9. These differences are reversed in foetal and adult haemoglobins of the cow, sheep, and goat, as is their resistances to alkali (Jonxis, 1949).

Electrophoresis of Foetal Haemoglobin

Andersch, Wilson, and Menten (1944) studied newborn infant and adult HbCO in *p*H 7.1, 0.2 M phosphate buffer and obtained a distinct separation of Hb-A and Hb-F and measured their mobilities. They were able to detect the small proportion of surviving Hb-F in a 90-day infant blood. Andersch *et al.* discussed the evidence due to Brinkman *et al.* and the results of earlier studies which pointed to the heterogeneity of adult human haemoglobin, and attempted to confirm this point by electrophoretic analysis; one adult blood studied by them showed a minor component with a mobility approaching that of Hb-F.

Andersch and his colleagues also observed a large difference by a factor of about 2 between the sedi-

mentation constants of Hb-A and Hb-F in the ultracentrifuge. Assuming identical diffusion constants, the difference corresponds to a molecular weight for Hb-A about twice that of Hb-F.

Hoch (1949, 1950) devised conditions for the electrophoretic testing of protein homogeneity and the detection of small differences in mobility. These conditions were utilized by Beaven *et al.* (1951) for analysing foetal and early infant human haemoglobins. Using a differential method (Hoch, 1949) the anodic mobility of Hb-A was found to be greater than that of Hb-F at both *p*H 7.1 and 8.0. Analyses of mixtures were made in 0.01M Na_2HPO_4; a low ratio of ionic strength: protein concentration, resulting in pronounced boundary anomalies, is favourable for showing up small proportions of a faster component as in the case of small amounts of Hb-A in foetal bloods (Hoch, 1950). By working under these carefully selected conditions Beaven *et al.* (1951) found the Hb-A content of foetal blood to be at least 6% at 20 weeks' foetal age, about 20% at birth, about 50% at two months and 80-90% at four months after birth. The ultra-violet absorption spectral difference between Hb-A and Hb-F discovered by Jope (1949) was utilized in this work to provide confirmatory analyses of bloods rich in Hb-F.

Rich (1952) successfully used somewhat similar conditions to analyse mixtures of Hb-A and Hb-F (as HbCO) in a study of Mediterranean trait and Cooley's anaemia, and confirmed Liquori's finding (1951) that Hb-F is present in Cooley's anaemia; in two of Rich's cases the Hb-F contents were nearly 100%. For newborn infant blood Rich found about 20% Hb-A in agreement with other workers.

Zinsser (1952) achieved the separation of Hb-A and Hb-F in phosphate buffer of higher ionic strength by using long periods of electrophoresis (15-20 hrs.), such as were employed with success in the study of sickle-cell haemoglobins (Pauling *et al.*, 1949). Zinsser obtained values for the mobilities of Hb-A and Hb-F at *p*H 6.94 to 7.00, and also observed differences between these two types of Hb with respect to (i) the change in mobility HbCO to Hb; (ii) the influence of various buffer ions on mobility; (iii) the effect of caprylate ion in producing a colourless component (not due to haem displacement), and (iv) the number of globin components after haem-removal by acid treatment.

Waris (1954) used the micro-electrophoresis apparatus of Antweiler (1949, 1952) to examine human haemoglobins from normal adults, newborn infants, and various types of anaemias in a variety of buffers at various *p*H values. He was unable to obtain an unequivocal separation of Hb-A and Hb-F, and no distinct variations from the Hb-A pattern were observed in the bloods from pernicious or other anaemias. At the highest currents used and over long periods convection effects led to splitting of the single peak. Waris's results emphasize the comments made by Hoch (1949, 1950) on the electrophoretic separation of proteins of nearly equal mobility, and the relative difficulty of the Hb-A/Hb-F separation.

Spectrophotometry of Hb-F

Jongbloed (1938) was unable to detect any difference in the ultra-violet absorption spectra of foetal and adult human haemoglobin. Lambrechts and Martin (1951) made a careful study by photoelectric spectrophotometry at 10A spectral bandwidth of a large number of foetal and adult human bloods and found the wavelengths of the maxima and minima in the visible to be identical for the two groups of specimens in both the HbO_2 and Hb states, as were the analytically important absorbence ratios to within approximately 2%. They were thus able to conclude with confidence that the various methods, based on the absorption characteristics in the visible, used for the estimation of Hb in adult bloods, were valid for newborn infant bloods.

The moving-plate, logarithmic-cam technique, devised by Holiday (1937, 1950) for the accurate location of fine-structure bands in absorption spectra, enabled Jope (1949) to demonstrate that the tryptophan fine-structure band in the globin absorption band was located at significantly different wave-lengths in foetal (2898 A) and adult (2910 A) human haemoglobins; by the moving-plate method the position of this fine-structure band can be found with a precision of $\pm$1A under favourable circumstances. Jope also showed by measurements on very thin layers of settled cell suspensions that this difference between Hb-A and Hb-F is also found when the haemoglobins are examined in intact erythrocytes. No such difference in the ultra-violet absorption spectra could be found for the foetal and adult haemoglobins of sheep and rat; the 2910 A band position was found to apply to the adult haemoglobins of several vertebrates, and it was also possible to show that in two mammalian muscle haemoglobins (myoglobins) the band was at 2900 A, comparable with the human foetal position.

Beaven *et al.* (1951) applied Jope's observation to the analysis of mixtures of Hb-A and Hb-F as encountered in foetal, full-term and early infant bloods by constructing a calibration curve relating the position of the tryptophan fine-structure band

to the proportion of Hb-F in the blood sample, as found by parallel electrophoretic analysis. This spectrographic method, we have found in subsequent work, is capable of detecting Hb-F in admixture with Hb-A down to a level of about 10%, with an average of precision of about ±10% over the range 20 to 80% Hb-F. This order of precision is useful for the examination of large numbers of bloods and the method is very rapid if the rather specialized spectrographic equipment required is available.

Rich (1952) devised an ultra-violet spectrophotometric method for the analysis of the Hb-A/Hb-F mixtures, based on Jope's observation, but adapted for commercial photoelectric instruments, which are now widely available in clinical chemistry laboratories. The Rich slope method is in principle applicable over the entire range of mixture composition with an estimated precision of ±6% and the results agree with electrophoretic analyses to within 7% (Rich, loc. cit.). In our experience the Rich slope method may be of greater potential utility than the Jope moving-plate method but entails very precise spectrophotometric technique and the basic calibration curve is not easy to establish with certainty.

The Oxygen Dissociation Curve for Foetal Blood

A number of investigators have established differences between the oxygen dissociation curves for foetal and adult blood, and also for the maternal blood in pregnancy. Leibson, Likhnitzky, and Sax (1936) found that the curve for the blood of normal women varied within narrow limits whilst the maternal blood in pregnancy gave a curve displaced slightly to the right, probably through increased hydrogen ion concentration. The dissociation curve for foetal blood lay to the left of that for normal women. Essentially similar results have been found by several investigators (Haselhorst and Stromberger, 1931; Eastman, Geiling, and De Lawder, 1933; Darling, Smith, Asmussen, and Cohen, 1941), and the question is discussed in detail by Barcroft (1946).

It is generally considered that the difference in behaviour of the foetal corpuscles is best accounted for by the presence of a different haemoglobin, but the situation is complicated by possible differences in ionic environment within the corpuscle, permeability and blood *p*H. Haurowitz (1935) found that in dilute solution human foetal haemoglobin was unique in having a lower oxygen affinity than the maternal haemoglobin, and this reversal of the behaviour found in the corpuscles has been confirmed by Hill (see Barcroft, 1946) and by McCarthy (1943) for strong solutions of the haemoglobin. McCarthy (1943) found that the maternal corpuscles markedly decreased the affinity of the adult Hb, whereas the foetal cells had little effect on the foetal Hb, thus accounting for the position when the whole bloods were compared.

In the case of the bull frog, the transition between oxygen dissociation curves of tadpole and frog is particularly well seen. In the tadpole, the curve is hyperbolic, with efficient loading capacity for acquiring oxygen under water; the curve of the metamorphosed frog, respiring air in the lungs, is definitely sigmoid and efficient for unloading oxygen to the tissues (McCutcheon, 1936; Barcroft, 1946).

Oxidation of Foetal Haemoglobin to Methaemoglobin

An interesting question, which has been little explored, is the ease of oxidation of foetal haemoglobin to methaemoglobin. In blood of normal adults, methaemoglobin, or other pigment without oxygen-carrying power is present only to a very small extent (Ramsay, 1949; Wootton, 1950). Künzer and Savelsberg (1951) found 2.12% of methaemoglobin in the blood of suckling infants in the first three months, as against 1% in the blood of all other age groups; cord blood samples contained only 1%. They suggest that this relates to low activity of the glycolytic enzyme systems which normally prevent by reduction the intracorpuscular accumulation of methaemoglobin (see Barcroft, Gibson, and Harrison, 1949). Well water containing nitrates may cause methaemoglobinaemia in infants (Comly, 1945), and Cornblath and Hartmann (1948) considered that nitrite formed by reduction by microorganisms in the gastro-intestinal tract was responsible. Lecks (1950) obtained some evidence that more ready oxidation of foetal haemoglobin by nitrites also played a part. The frequent occurrence of heavy methaemoglobinaemia in premature infants treated with sulphamezathine (Mann and O'Brien, 1951) may also indicate this. Studies are needed on systems free of enzymes, or in the presence of inhibitors.

Betke (1953) has measured the rate of oxidation of cord-blood and adult, purified haemoglobins to methaemoglobin by sodium nitrite at *p*H 5.5, and found that the former is transformed at about twice the rate of the latter.

Clinical Significance of Foetal Haemoglobin

It is usual to point to the different sites of erythrocyte formation in foetus and adult in explaining the two types of haemoglobin. The liver as

opposed to the marrow is often quoted as the principal site of origin for foetal erythrocytes (see Barcroft, 1946; Drabkin, 1951). However, foetal haemopoiesis is a complicated process, passing through several morphologically distinct phases, and widely distributed in many organs and tissues (Gilmour, 1941). The liver is the chief site in mid-foetal life, but skeletal sites appear in the clavicle of the 43 mm. embryo (Gilmour, 1941), and the marrow progressively assumes the principal role from the fifth month of intra-uterine life onwards, and is the exclusive site from 15 to 21 days after birth (Gilmour, 1941).

The spectrophotometric data of Beaven *et al.* (1951) indicate a very gradual change-over from foetal to adult-type haemoglobin formation, beginning not later than the middle of intra-uterine life. Even haemoglobin from a 20-week foetus contained 6–7% adult-type Hb, and at term about 20% was present. It is possible that the foetal-type globin is an expression of the general foetal anabolism rather than the specific site of origin. These workers found that no more than 10% of foetal haemoglobin persists four months after birth, and this would be compatible with the morphological observations of Gilmour (1941) that erythropoiesis is entirely intramedullary from 15 to 21 days after birth, but also indicates that only adult haemoglobin is formed after delivery. Other workers suggest that foetal haemoglobin disappears in the first few months of life (Trought, 1932; Haurowitz, 1935), but Singer *et al.* (1951a) using a denaturation test claim to have detected a small percentage persisting as long as two years after birth in healthy infants. It is clear that further correlation is required between the morphology of haemopoiesis during intra-uterine life and infancy, and the detection of foetal haemoglobin by a range of physical methods.

It has frequently been considered that foetal haemoglobin may persist after birth or reappear in various pathological conditions (see Barcroft, 1934) or modified environment conditions (e.g. in dwellers at high altitudes; Rubowitz, 1933). In pernicious anaemia, the similarities between megaloblastic haemopoiesis and the earliest, primitive generation of nucleated red cells in pre-somite embryos and also the part played by the liver have prompted several investigators to look for foetal haemoglobin. Schenck (1930) thought alkali-resistant haemoglobin occurred in this condition. Larsen (1951) also claimed that foetal haemoglobin occurred, and might be responsible for the thin macrocytes present in air-dried films of the blood; such cells did not disappear after treatment by purified liver extracts, folic acid, or vitamin B12. However, Trought (1932) and Haurowitz (1935) have found the rapid denaturation characteristic of normal adult Hb, and Jope and O'Brien (1949) prepared orthorhombic crystals of normal adult HbO_2 from pernicious anaemia blood.

A number of Italian workers have noted the presence of alkali-resistant haemoglobin in the red cells in thalassaemia major (Bianco, 1948; Putignano and Fiore-Donati, 1948; Sansome and Cusmano, 1950; Vechio, 1948). Liquori (1951) investigated the blood in thalassaemia major (Cooley's anaemia; Cooley and Lee, 1925), and obtained unequivocal evidence for the presence of a mixture of adult and foetal haemoglobins. This was indicated by the slow alkali-denaturation rate, and by the absorption method. Crystals of HbO_2 were prepared with the morphology and higher solubility characteristic of the foetal pigment. Rich (1952) by the ultra-violet absorption method has consistently found large amounts of foetal haemoglobin in the blood of children with thalassaemia major whereas the parents with thalassaemia trait have the adult pigment. Genetically, Cooley's anaemia appears to be a homozygous condition inherited from heterozygous parents (Moncrieff and Whitby, 1934; Valentine and Neel, 1944), and a fundamental defect in the ability to synthesize haemoglobin occurs, the presence of foetal Hb being the result rather than the cause of this. In Fig. 1 the presence of foetal haemoglobin is shown in the blood of a Cypriot child with Cooley's anaemia, whose parents with Mediterranean trait have the adult pigment only. Roche, Derrien, Diacono, and Roques (1953) have studied 21 cases of different degrees of thalassaemia occurring in individuals of various racial origins (Arabs, Maltese, Sicilians) all living in Tunis. The individuals with thalassaemia minima had haemoglobin indistinguishable from normal adult haemoglobin, as studied by the solubility curves of HbCO in phosphate buffers and by alkali denaturation. The cases with thalassaemia minor (Rietti-Greppi-Micheli disease) had 15 to 40% haemoglobin behaving like the foetal pigment in these tests, whereas the cases of thalassaemia major (Cooley anaemia) had 69–98% of foetal-type pigment.

Singer *et al.* (1951a) have detected foetal haemoglobin in Cooley's anaemia, in the trait in small amounts, and also they believe that small amounts of alkali-resistant pigment may occur in a variety of haematological disorders, including some cases of acute and chronic leukaemia, pernicious anaemia, leuco-erythroblastic anaemia due to secondary carcinomatosis of the marrow, chronic aregenerative anaemia, and hereditary spherocy-

tosis; haemorrhagic and iron-deficiency anaemias have normal adult pigments only.

Haemoglobins from a series of haemolytic anaemias have been studied by Beaven and White (1953) by the alkali-denaturation method and by ultra-violet spectrophotometric methods. The series included the cases of atypical congenital haemolytic anaemia published by Dacie, Mollison, Richardson, Selwyn, and Shapiro (1953), as well as hereditary spherocytic anaemias. Despite the frequent occurrence of profoundly disturbed haemopoiesis, including the existence of extramedullary blood formation, the haemoglobin appeared to be predominantly of the adult type. In certain cases associated with the presence of sickle-cell haemoglobin, however, variable amounts of foetal haemoglobin were found (*vide infra*). Beaven and White (1953) failed to find foetal haemoglobin in pernicious anaemia of moderate or severe degree, or in most cases of acute or chronic leukaemia in adults and children. Acute erythro-leukaemia in a child of 2 years and subacute erythro-leukaemia in an adult were also associated with adult-type haemoglobin. On the other hand, two slowly progressing leukaemic processes in boys of 3 and 4 years respectively were associated with amounts of foetal haemoglobin which fluctuated from time to time, but reached as high as 35% of the total haemoglobin. A short series of primary and secondary polycythaemias have not yielded evidence for the presence of foetal haemoglobin.

SICKLE-CELL DISEASE AND SICKLE-CELL HAEMOGLOBIN (HAEMOGLOBIN S)

Understanding of sickle-cell disease has advanced rapidly in recent years on chemical, physical, and genetic grounds. A characteristic, abnormal haemoglobin has been recognized, playing a crucial part in the physical changes which distort the erythrocytes on reduction of oxygen tension to form the sickle cell. It is becoming clear that the formation of haemoglobin S is under the control of a dominant gene, and that in the heterozygous condition this leads to formation in the erythrocytes of a mixture of normal and sickle-cell haemoglobins characteristic of the "sickle trait" or sicklaemia, whereas in the homozygous state a large excess of sickle haemoglobin occurs in the erythrocytes and the severe manifestation of sickle-cell anaemia occur. On the basis of gene-controlled haemoglobin synthesis, the gene for sickle-cell haemoglobin may interact with genes for other abnormal haemoglobins (C or D) or with the thalassaemia gene, and the affected progeny suffer a variety of haematological and haemolytic disorders related to sickle-cell disease.

The first important discovery in this field was that of Pauling *et al.* (1949). These workers carried out electrophoretic analysis in the Tiselius apparatus on the haemoglobins from normal individuals and from patients with sickle-cell anaemia, and found a characteristic difference in behaviour.

Electrophoretic Properties of Sickle-cell Haemoglobin

Working with both HbCO and Hb (stabilized with dithionite) in 0.1 ionic strength phosphate buffers over the *p*H range approximately 5.5 to 8.5, Pauling *et al.* obtained distinctly separated plots of mobility against *p*H for normal adult and sickle-cell anaemia haemoglobins, corresponding to a difference in isoelectric point of 0.22 to 0.23 *p*H units. In a cacodylate buffer, *p*H 6.52, ionic strength 0.1, the mobility of sickle-cell anaemia HbCO exceeded that of normal adult HbCO by 0.40×10^{-5} (units are $cm.^2\ sec.^{-1}\ volt^{-1}$). The mobility difference between Hb – S and Hb – A measured by Pauling *et al.* corresponds to two to four more net positive charges per molecule for Hb – S in the isoelectric *p*H region.

With sicklaemia (sickle-cell trait) bloods, separation into two components, one of which was electrophoretically identical with normal adult HbCO and the other with that of sickle-cell anaemia, were obtained. Analyses of this two-component system, checked against artificial mixtures of normal and sickle-cell anaemia bloods, gave a figure of about 40% for the proportion of the abnormal or sickle-cell haemoglobin (Hb – S) in sickle-cell trait. This figure is important for the genetic analysis of sickle-cell characteristics, as it corresponds to the heterozygous condition.

In their first studies, Pauling *et al.* (1949) considered the haemoglobin in sickle-cell anaemia to be uniformly Hb – S, whereas in the sickle-cell trait Hb – A and Hb – S occurred together in the approximate proportion 60:40. Greater activity of the allele controlling Hb – A synthesis over that for Hb – S synthesis was thought to explain the preponderance of the former. Wells and Itano (1951) carried out further analyses in the Tiselius apparatus and confirmed that in the sickle-cell trait the mean proportion of Hb – S was 37.5%, though the range in 42 individuals was from 24 to 45%. The amount for any individual fluctuated only very slightly with time. Some patients with sickle-cell anaemia who had remained sufficiently well to dispense with the need for blood trans-

fusions over long periods were examined also. These individuals had 5 to 20% of apparently normal haemoglobin in addition to Hb–S. Under the conditions of analysis employed Hb–S and Hb–F would not have been distinguished, and subsequent work has established that foetal haemoglobin may accompany Hb–S in sickle-cell anaemia under various conditions.

Paper Electrophoresis

Spaet (1953) applied the very simple technique of Durrum (1950) to the analysis of haemoglobin mixtures and obtained useful separations of Hb–A/Hb–S, Hb–A/Hb–C, and Hb–S/Hb–C mixtures in veronal buffer, *p*H 8.6. Hb–A and Hb–F could not be differentiated. The Hb zones were visualized by staining with bromphenol blue.

Smith and Conley (1953) have described the paper electrophoresis of human haemoglobins with special reference to the detection of Hb–C. They have used apparatus similar to that of Kunkel and Tiselius (1951) and Slater and Kunkel (1953), and veronal buffer, *p*H 8.6, ionic strength about 0.06 and achieve good separations of Hb–C and Hb–S from Hb–A in four hours' running or less, the mobilities decreasing in the order given. With running times extended to six hours they also claim a separation of Hb–A and Hb–F, but this is apparent in their diagrams only to the extent of an enlargement of the Hb–A spot by the slightly slower running Hb–F; the two types of Hb do not give resolved spots (cf. Reynaud, 1953). Schneider (1953) has also used paper electrophoresis successfully for distinguishing between normal, sickle-cell and C haemoglobins, using veronal buffer at *p*H 8.6 to 9.

The Solubility of Sickle-cell Haemoglobin

Sickled cells are birefringent (Sherman, 1940), suggesting orderly molecular arrangement of their contents. Perutz and Mitchison (1950) were impressed by the similarities between the optical properties in polarized light of sickled red cells and of small crystalline plates and needles of normal reduced haemoglobin. Such features as pleochroism, extinction directions, anomalous colours on compensation, and dispersion of the birefringence in the neighbourhood of absorption bands all corresponded. They then showed that although the solubilities in strong phosphate buffers were similar for the HbO_2 compounds of normal and sickle-cell haemoglobins, the reduced haemoglobins differed completely. Normal reduced haemoglobin has about the solubility of HbO_2, but the fall in solubility of reduced Hb–S is very profound, the solubility being only about one hundredth of the oxy-compound under equivalent conditions. Perutz *et al.* (1951) later confirmed this work, using crystallized preparations of Hb–A and Hb–S, and also showed that the same distinction in the solubilities of the reduced proteins obtained in solutions of physiological salt concentration as well as in strong buffers.

Determination of the solubility of the reduced haemoglobin in phosphate buffers of high ionic strength provides valuable means for detecting Hb–S alone or in mixtures. The net solubility of the reduced haemoglobin for sickle-cell trait red cells is intermediate between that for Hb–A and for pure Hb–S, and similar results are obtained when Hb–S occurs in conjunction with appreciable amounts of other abnormal haemoglobins (Hb–F, C or D) (Itano, 1953a and c; Beaven and White, 1953). However, where mixtures occur, the features determining solubility are complex, and the procedure needs to be used with care. In sickle-cell trait, the results of Itano (1953a and c), using a system containing 1% w/v of haemoglobin, are similar to those of Beaven and White (1953) at comparable ionic strength ($r/2=4.75$), using a system with 0.4 to 1% w/v of haemoglobin. But Dr. N. A. Barnicot (1954) has pointed out to us that large deviations occur on varying the amount of haemoglobin, net solubility rising with increase in added protein. Obviously, the degree of interaction in mixtures will be of great importance in determining the result. In Fig. 2, the results of solubility studies on reduced haemoglobins are given as regression lines, fitted to the experimental values at various ionic strengths for normal and non-sickling haemoglobin samples, and for sickle-cell trait and sickle-cell anaemia samples.

Vandepitte and Louis (1953) have described filamentous processes derived from sickled cells, occurring in wet-preparations of aspirated marrow from sickle-cell anaemia patients, but not from the trait. These processes are presumably tactoids derived from cells which sickle at the reduced oxygen tension of the marrow sinusoids, and become trapped.

Gel-formation by Reduced Hb–S

Information of a different character about the state of reduced haemoglobin in the sickled red cell was obtained by Harris (1950), who found that stroma-free, concentrated solutions of Hb–S became very viscous on exposure to low oxygen tensions, and that under the phase-contrast microscope tactoids resembling sickled cells were visible. Tactoids are elongated bodies formed by various molecules capable of aggregation or polymerization, in solution, and, although not as organized

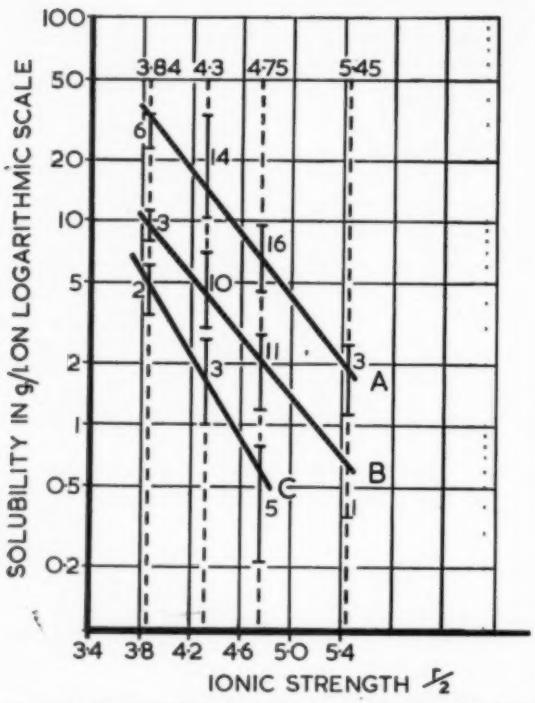


FIG. 2.—Solubility of reduced haemoglobin in *p*H 6.7 phosphate buffer at high ionic strengths. Regression lines are fitted to experimental values for normal and non-sickling (A), sickle-cell trait (B), and sickle-cell anaemia (C) groups. The range of values for each group, at the various ionic strengths of buffer employed, is shown in the vertical bars, and the number of observations is indicated for each.

as a crystal, possess sufficient molecular orientation to confer birefringence in polarized light (Zocher, 1925). Harris (1950) concluded that the sickling phenomenon was the result of tactoid formation. Whether the sickled cell is to be regarded as containing crystals or tactoids of reduced haemoglobin is not settled, but recent observations on the gelling of sickle-trait haemoglobin is of considerable interest in this connexion.

Singer and Chernoff (1952) found that highly concentrated solutions of sickle-trait haemoglobin gelled on long exposure to CO_2 in the absence of oxygen. The gelling affected the entire solution, and tactoids were not visible. The proportion of Hb–S was relatively low, and it was thought the interaction with the Hb–A must occur for gel formation. Singer and Singer (1953) find that gelling of reduced haemoglobin is entirely indicative of the presence of Hb–S. The concomitant presence of normal adult haemoglobin or of Hb–C lowers the amount of Hb–S necessary for the phenomenon, and interaction between the molecules to form polymeric gels. On the other hand, foetal haemoglobin has no such effect in promoting gel-formation. The "lowest gelling point," expressed as the minimal concentration of total pigment at which gelling can be elicited, is lowest for sickle-cell anaemia haemoglobin, highest for sickle-cell trait haemoglobin and intermediate for sickle-Hb C disease, thus providing a means of distinguishing between these conditions (Singer and Singer, 1953).

Other Characteristics of Sickle-cell Haemoglobin

Perutz *et al.* (1951) found that Hb–A and Hb–S were identical in the *x*-ray crystallographic properties of their derivatives, but with one exception. A unique form of orthorhombic HbO_2 was obtained from sickle-cell haemoglobin with a very large unit cell, in which the eight molecules were probably associated as dimers (Drabkin, 1951).

Amino-acid Composition.—Schroeder, Kay, and Wells (1950) analysed the globins of normal and sickle-cell anaemic negroes by fractionation of the hydrolysis products on starch partition-fractionation columns, in which approximately 98% of the total weight and N of the proteins were accounted for. The two globins were found not to differ with respect to their contents of basic and acidic amino-acids; the Hb–S globin probably contained slightly less leucine and more serine and possibly less valine and more threonine. These differences cannot directly contribute to the net charges on the proteins and therefore influence their electrophoretic properties (which differ significantly, cf. Table 2), though Schroeder *et al.* suggest that they may affect the folding or coiling of the polypeptide chains and hence alter indirectly the disposition of charged groups in the proteins. Dickman and Moncrief (1951) found 26 primary amide groups per molecule in both Hb–A and Hb–S.

Havinga (1953) compared the globins of normal adult human and sickle-cell anaemia haemoglobins and found them to be essentially identical with respect to optical rotary power and terminal amino-acid residues (four valine residues/mole for both Hb–A and Hb–S). The splitting of the HbCO derivatives into haem and globin by treatment with acid acetone (Anson and Mirsky, 1930) was more difficult with Hb–S than with Hb–A, suggesting a

difference between the two globins with respect to the haem-globin linkage, as postulated earlier by Pauling *et al.* (1949). Havinga also obtained an indication that Hb–S might contain a methionine end-group.

TABLE II

SOME PROPERTIES OF HUMAN HAEMOGLOBIN VARIANTS

Haemoglobin Type	Iso-electric Point (Itano, 1953a)	Anodic Mobility Relative to HbA by Paper Electrophoresis at *p*H 8·6	Solubility of Reduced Haemoglobin
Normal, A ..	6·87 (6·9 estimated from Zinsser, (1952)	—	High *M* 6·8g./l.; 4·45–9·3, r/2 4·75 (Beaven and White, 1953). 1·12–2·48 (Beaven and White, 1953); 1·39 ± 0·15 (Itano, 1953c). r/2 5·45
Foetal, F ..	6·98 (Estimated from Zinsser, 1952)	Slightly less	Higher than A
Sickle-cell, S.	7·09	Less	Very low *M*0·66g./l.; 0·21–0·78, r/2 4·75 (Beaven and White, 1953) 0·1–0·95, r/2 4·75 (Itano, 1953c).
Haemoglobin C		Less than S and D	High
Haemoglobin D	7·09	Less Same as HbS	High
Haemoglobin E (Itano, *et al.*, 1954)		Between A and S by free Electrophoresis (Itano *et al.*, 1954).	High (Itano *et al.*, 1954).

Sulphydryl Groups in Hb–S.—Ingbar and Kass (1951), following up an indication that chemical inhibitors of the sickling process were also known SH group inhibitors, determined the SH-group content of normal adult, sickle-cell anaemia and sickle-cell trait haemoglobins by amperometric titration in 0.85% saline with 0.001N silver nitrate. They found 2.04 ± 0.04 SH group per mole (68,000) in normal adult haemoglobin in agreement with Hughes (1949) and $3.07 \pm .16$ groups per mole in sickle cell anaemia haemoglobin. The two sickle-cell trait bloods studied gave an intermediate value, 2.46 ± 0.03, equivalent to about 46% Hb–S in admixture with Hb–A. Ingbar and Kass pointed out that their results did not distinguish between a difference in total SH group content or a difference in reactivity of the SH groups of Hb–A and Hb – S; they found, however, that the SH groups of both types of Hb were blocked stoichiometrically by p-chloromercuribenzoate.

Resistance of Sickle Cells to Heinz Body Formation.—Fisher (1953a) reported the formation of Heinz bodies in non-sickling adult and infant erythrocytes by exposure to 2% aqueous bisulphite (metabisulphite) but not in sickle-cell anaemia or sickle-cell trait erythrocytes under the same conditions, and suggested that the presumptive denaturation of globin associated with Heinz body formation involved reactive groups (possibly –SH) which were not available to the above reagent in Hb–S; this suggestion is consistent with the differences in polypeptide chain configuration between Hb–A and Hb–S envisaged by Havinga and Itano (1953). Normal haemoglobin readily enters into a complex oxidation-reduction reaction with phenylhydrazine (Beavan and White, 1954) but our preliminary observations suggest that Hb–S is much less active in this respect also than is Hb–A.

Foetal Haemoglobin in Sickle-cell Anaemia

Using alkali-denaturation techniques, Singer *et al.* (1951a) found that 2–24% of resistant haemoglobin frequently occurred in the erythrocytes of sickle-cell anaemia but not in the trait. Combining this test with that of tactoid formation for sickle-cell haemoglobin, Singer and Chernoff (1952) recognized normal adult Hb–A and sickle-cell Hb–S in the trait cells, and showed that in sickle-cell anaemia the haemoglobin S might be accompanied by alkali-resistant, foetal-type Hb–F (Fig. 1).

The presence of foetal haemoglobin in sickle-cell anaemia has also been established by Itano (1953a and b) by the alkali-denaturation and spectrophotometric methods, and by solubility measurements. The presence of foetal haemoglobin in the sickle-cell increases the net solubility (Itano, 1953a, b, c) which is advantageous to the individual. There is also some evidence that sickling is difficult to elicit in the young infants of sicklaemic parents, possibly due to a persistence of foetal haemoglobin (Watson, 1948). In this connexion the failure of Hb–F to enhance the gelling power of the Hb–S by interaction is of interest (Singer and Singer, 1953).

Singer and Fisher (1952) have followed survival of transfused sickle-cell anaemia red cells containing 12–18% of Hb–F. Evidence was obtained that Hb–F persisted longer than Hb–S in the circulation of recipients, and that sickle-cell anaemia patients may have three classes of red cells containing (*a*) Hb–S only, (*b*) a mixture of Hb–S and Hb–F and (*c*) cells mainly of Hb–F. The life span of the three classes is considerably shortened in (*a*), intermediate in (*b*), and longest in (*c*). Singer and Fisher (1953a) suggested that for practical purposes

the proportion of Hb–S in sickle-cell anaemia blood might be expressed as 100% less the proportion of Hb–F found by alkali denaturation. In two cases at least, however, they found evidence for 10–20% Hb–A accompanying about 10% Hb–F.

The Genetics of Sickle-cell Anaemia

Neel (1951, 1952) has postulated that the sickling phenomenon is due to a gene, which in single dose produces the trait, and in double dose, the homozygous state with development of sickle-cell anaemia. This is well supported by the chemical evidence for a mixture of sickle-cell Hb and slight excess of normal Hb in the trait cells, but an excess of sickle-Hb with perhaps some foetal Hb, in the anaemia. The theory requires that both parents should carry the gene, and their blood sickle. This is largely true, but exceptions occur. Singer and Fisher (1953b) have shown that in some anomalous cases the erythrocytes of the non-sickling parent may contain no more than 5% of sickle-cell Hb detectable only by careful electrophoretic analysis. The investigations of Singer and Singer (1953) also suggest that gelling may not occur where sickle-cell trait haemoglobin contains less than about 20% of Hb–S, and that under these circumstances failure of the red cells to sickle under test conditions may be expected.

In some cases it appears that one parent has the sickle trait, the other the thalassaemia trait (Cooley or Mediterranean trait), and that although the genes for the two conditions are separate, they may interact to produce a haemolytic syndrome with features of both sickle and Mediterranean anaemias in some of the offspring (Powell, Rodarte, and Neel, 1950; Sturgeon, Itano, and Valentine, 1952). This condition was first described in Sicilian and southern Italian families by Silvestroni and Bianco (1952) and called micro-drepanocytic disease. The interaction of the gene for Hb–S with those for two further haemoglobins (C and D) leads to a further proportion of apparently anomalous cases of sickle-cell anaemia (*vide infra*).

Since sickle-cell anaemia is a serious disease with a high mortality rate, it might be expected that the gene would be gradually eliminated. This does not appear to be so, and there is some evidence that a high proportion of persons with sickle-cell trait may be maintained in various African populations by virtue of their greater resistance to parasitic infection (Raper, 1949), particularly to malarial infection (Brain, 1952; Beet, 1946; Allison, 1954). Allison's data for Luo tribesmen in East Africa indicates that possession of the sickle-cell trait is protective against *Plasmodium falciparum* infection in particular. Allison (1954) and Lehmann (1953) suggest further that the high proportion of foetal haemoglobin in young infants' cells is responsible for their relative resistance to malarial parasitization compared with older children in whom normal Hb–A has replaced Hb–F.

HAEMOGLOBINS IN HAEMOLYTIC ANAEMIA DUE TO SICKLE-CELL : THALASSAEMIA INTERACTION

Among families of central and southern Italian and Sicilian stock, Silvestroni and Bianco recognized examples of the thalassaemia syndromes, and of sickle-cell trait and anaemia. They also described a condition which appeared to share genetic characters of both groups, which they called microdrepanocytic disease, and which appeared in families where one parent carried the sickle-cell trait and the other the Mediterranean trait (see Silvestroni and Bianco, 1952). A similar condition has been described in America, chiefly among families of Mediterranean origin (Powell *et al.* 1950; Sturgeon *et al.* 1952; Neel, Itano, and Lawrence, 1953) and in Greece (Caminopetros, 1952).

In thalassaemia, genetic interference with the synthesis of normal adult haemoglobin may occur (Rich, 1952), the extent of interference being much greater in the homozygous thalassaemia major than in the heterozygous minor condition; the Hb–F which is present to a variable extent may be regarded as compensatory. In sickle cell-thalassaemia disease genes for both diseases are present, possibly in a doubly heterozygous condition (Silvestroni and Bianco, 1952). Hb–S is synthesized in considerable excess (Sturgeon *et al.*, 1952), and Hb–A and Hb–F may be present in addition (Itano, 1953b).

The reviewers have observed 20% of Hb–F and 80% Hb – S in the red cells of an Arab woman with this condition. (This sample was kindly made available by Dr. N. A. F. Young from Kuwait.) The condition has been observed in this country in a child, whose father is of West African descent and has sickle-cell trait, and whose mother is from Naples and has Mediterranean trait (Humble, Anderson, White and Freeman, 1954). The patient's haemoglobin appears to be predominantly Hb – S, and Hb – F has not been found.

Ascenzie and Silvestroni (1953) have shown that the high content of Hb–S in microdrepanocytic disease is associated with birefringence of the sickled red cells, and with the formation of birefringent tactoids in reduced concentrated solutions of the haemoglobin.

HAEMOGLOBIN C

Haemoglobin C and D are two further abnormal haemoglobins that have been well characterized, and are of importance since they occur particularly in the negro races, and genetic interaction with sickle-cell occurs, resulting in haemolytic anaemias of moderate degree.

The first of the haemoglobins, now called haemoglobin C, was described as haemoglobin III by Itano and Neel (1950) and Kaplan, Zuelzer, and Neel (1951). In certain negro families, children were found to suffer from a rather mild haemolytic disorder, with sickling of the red cells on reduction. Free electrophoretic analysis revealed that the haemoglobin of these children comprised a mixture of Hb–S and the hitherto unrecognized pigment with greater mobility than either Hb–A or Hb–S. In each case, one parent carried sickle-cell trait, and the other possessed trait characteristics for the new haemoglobin, their red cells containing Hb–A and Hb–C.

Numerous examples of the haemoglobin-C trait and of haemoglobin C–sickle disease have now been studied (Kaplan *et al.* 1953; Lehmann and Edington, 1954). The trait condition appears harmless, though small numbers of target cells and slightly hypochromatic red cells may be present in the blood (Ranney, Larson, and McCormack, 1953), and Smith and Conley (1953) found an incidence of 2% among 500 American negroes, whereas Hb–S was encountered in 8.4%. Larson and Ranney (1953) found 56% of Hb–A and 44% of Hb–C in the trait. The homozygous condition, with predominance of haemoglobin C, has been described by Ranney *et al.* (1953), Spaet, Alway, and Ward (1953), and Levin, Schneider, Cudd, and Johnson (1953). Although not associated with clinical anaemia, the homozygous individuals exhibit slight reticulocytosis and numerous target cells in the blood, decreased fragility and absence of sickling, and splenomegaly.

Among the offspring of parents with sickle-trait and haemoglobin C trait, individuals appear with a mixture of Hb–S and Hb–C; the two haemoglobins appear to be rather evenly balanced in amount, and Larson and Ranney (1953) give 48% Hb–S and 52% Hb–C. These individuals often have a definite haemolytic process of mild degree, with moderate reticulocytosis and bilirubinaemia, and numerous target cells in the blood smear. There is a distinct tendency to increased haemolysis during pregnancy, and vascular lesions occur (Smith and Conley, 1953). In their studies of offspring from matings of sickle trait × Hb–C trait, Neel, Kaplan, and Zuelzer (1953) found the ratios for Hb–C trait: sickle-cell trait: normal: haemoglobin-C-sickle cell disease to be 4:1:5:2.

In a study of various physico-chemical properties of apparently homogenous haemoglobin C, Ranney *et al.* (1953) found the only distinction from Hb–A to lie in the difference of electrophoretic mobility. Since there is a considerable difference in mobility between Hb–A and Hb–C, and Hb–S is intermediate in behaviour, paper electrophoresis is particularly suitable for demonstrating this group (Spaet, 1953; Smith and Conley, 1953). In Fig. 3 Hb–S and Hb–C are demonstrated in the blood of a Jamaican woman resident in this country.

Itano (1953c) has studied the solubility of the reduced haemoglobin, and found it higher than normal in one homozygous specimen and eight trait samples, whilst considerable lowering of solubility was found in cases of haemoglobin C-sickle-cell disease, intermediate between values for sickle-trait and pure sickle-cell haemoglobin.

HAEMOGLOBIN D

In 1934 Cooke and Mack described a family apparently of white American ancestry, in which the father and two young children all exhibited slow sickling of the red cells on reduction. The children both had haemolytic anaemia, and an enlarged spleen was removed from the younger without effect.

In 1951, Itano re-examined the mother and two affected offspring, confirming that the red cells of the latter could sickle. On free electrophoresis of the haemoglobins, the offspring yielded uniform components with the mobility of Hb–S, and the solubility of the reduced haemoglobin was considerably lowered, approaching that of Hb–S. The red cells of the mother, and of a non-anaemic brother and sister, could not be sickled, but on free electrophoresis separation occurred into two components, resembling the Hb–A and Hb–S fractions of sickle-cell trait blood. However, the solubility of the reduced haemoglobin in these three individuals was close to normal, not lowered as in sickle-cell trait.

Itano (1951) called this new haemoglobin, with electrophoretic mobility resembling Hb–S but normal solubility when reduced, haemoglobin D. He considered that it occurred in the heterozygous trait condition in the mother, and was similarly inherited in the two unaffected offspring. In the two affected offspring, however, genes for synthesis of Hb–S and Hb–D were inherited from father and mother respectively. Further work (Itano, 1953c) has confirmed the solubility properties in Hb–D trait and Hb–D—Hb–S disease, and in the latter condition Itano (1953a) has also detected Hb–F.

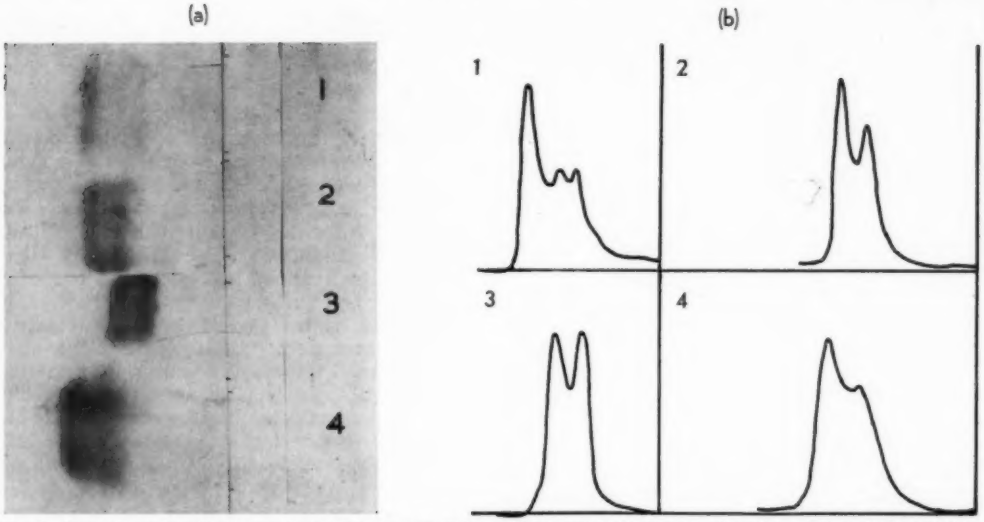


FIG. 3.—Paper electrophoresis of human haemoglobins run as HbCO on Whatman No. 1 paper strips 24 × 9 cm., *p*H 8.6, barbitone buffer, r/2 0.05. 210 v. applied at 5 ma. for 18 hours. (*a*) Strips photographed unstained; (*b*) tracings of strips after staining with naphthalene black and scanning by automatically recording densitometer (Lawrence, 1954). 1 shows normal haemoglobin mixed with haemoglobins from sickle cell—haemoglobin C disease. Order of peaks, left to right, normal, sickle-cell, and C haemoglobins. 2 shows haemoglobin D trait: normal and D haemoglobins. 3 shows sickle-cell—haemoglobin C disease. 4 shows sickle-cell trait with normal and sickle-cell haemoglobins. The mobility of sickle-cell and D haemoglobin is similar.

Itano (1951) considers that Hb – S and Hb – D interact on reduction, with lowering of the net solubility which may be greater in degree that that for simple sickle-cell trait; this may account for the ready destruction of the cells in the patients' circulation.

On paper electrophoresis, the similarity in behaviour between Hb–S and Hb–D is maintained. In Fig. 3, paper electrophoretic analysis is illustrated for a European woman carrying Hb–D trait, together with the analysis of her husband, carrying sickle-cell trait. The solubilities of the reduced haemoglobins are respectively normal and lowered to the extent found in sickle-cell trait. One child in the family has haemolytic anaemia, and paper electrophoresis reveals a large component resembling Hb–S while the solubility on reduction is within the range found in sickle-cell trait.

HAEMOGLOBIN E

Itano, Bergren, and Sturgeon (1954) have recently reported the binding of a fourth abnormal haemoglobin (Hb–E) in the red cells of a child with an atypical anaemia. The electrophoretic mobility of Hb–E was greater than that of Hb–A but slightly less than that of Hb–S. The ultra-violet absorption spectrum, solubility, and alkali-denaturation characteristics were similar to those of Hb–A. The other constituent of the haemoglobin from this patient was identified as Hb–F, and formed 41% of the total.

CONCLUSION

Striking progress has been made in recent years in the study of the biochemical defects underlying hereditary anaemias of the sickle-cell, thalassaemia, and related and inter-connected groups. A series of abnormal human haemoglobins is now firmly recognized, in addition to normal adult and foetal forms. Information on their genetic, haematological, and clinical implications is rapidly being assembled. The methods used in characterization of these haemoglobins are based on the fundamental physico-chemical procedures of protein chemistry. Some of these methods must remain as specialized techniques, but others, such as paper electrophoresis and photocolorimetric determination of alkali-denaturation rates, can be easily applied in the clinical laboratory.

Apart from the recognition of abnormal derivatives of chemically modified normal haemoglobins by spectroscopic procedures and investigation of pyrrol pigment metabolism, the variants of haemoglobin should be searched for in any obscure haemolytic anaemia in which diagnosis is not established by the conventional haematological procedures and on clinical grounds. This is particularly so where sickling of the red cells can be elicited, or the bloods film presents features such as numerous target cells.

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A FAMILY ILLUSTRATING THE DOUBLE INHERITANCE OF THE SICKLE CELL TRAIT AND OF MEDITERRANEAN ANAEMIA

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Application of the precise methods of physical chemistry to medical problems frequently provides an exact explanation of hitherto imperfectly understood phenomena. This is well illustrated by the recent advances made in the understanding of sickle cell anaemia and the sickle cell trait. In 1949, Pauling, Itano, Singer, and Wells demonstrated that the carbon monoxide derivative of the haemoglobin derived from the red cells of sickle cell anaemia possessed a different mobility from normal haemoglobin. They showed that the red cells of sickle cell anaemia contained almost entirely this "sickle" haemoglobin, whilst the red cells of sickle trait carriers contained about 50% sickle haemoglobin and 50% normal haemoglobin. In 1950, Perutz and Mitchison demonstrated that reduced sickle haemoglobin was extremely insoluble and the crystals formed were similar to red cells that had undergone "sickling." Thus sickle cell haemoglobin joined normal haemoglobin and foetal haemoglobin as a separate variety of human haemoglobin. In 1951 (a and b) Singer, Chernoff, and Singer demonstrated up to 24% foetal-type haemoglobin in sickle cell anaemia but did not find any in 60 sickle trait carriers. Foetal haemoglobin was also found in other forms of inherited haemolytic anaemias, especially in the severe forms of Mediterranean anaemia. This latter finding was confirmed by Liquori (1951) who found between 40 and 60% of foetal haemoglobin in seven cases of the homozygous form of Mediterranean anaemia (Cooley's anaemia). In sickle trait carriers 24 to 45% of the haemoglobin is usually found as sickle haemoglobin (Wells and Itano, 1951). These authors mention anomalous sickle cell anaemia cases with 80 to 95% sickle haemoglobin and 5 to 20% normal haemoglobin. Singer and Fisher (1953) found a family of sickle trait carriers with so little sickle haemoglobin (5%) that the red cells would not sickle. Two other separate haemoglobins have also been discovered, haemoglobin III or C (Kaplan, Zuelzer, and Neel, 1951) and haemoglobin D (Itano, 1951). There are thus five varieties of human haemoglobin: normal adult or A, foetal or F, sickle haemoglobin S, and haemoglobins C and D (National Institutes of Health, 1953) *

By combining the results obtained by electrophoresis and by quantitative alkali denaturation (Singer) the red cells of sickle cell anaemia are usually found to contain sickle haemoglobin 76 to 100% and foetal haemoglobin 2 to 23% only. The red cells of the sickle carrier contain sickle haemoglobin 5 to 45% and 95 to 55% normal A haemoglobin. The red cells of the severe (homozygous) form of Mediterranean anaemia contain normal adult haemoglobin with from 5 to 60% foetal haemoglobin. The mild (heterozygous) form of Mediterranean anaemia usually contains only adult haemoglobin in their red cells. Intermarriage between Mediterranean anaemia and sickle cell families has been described in Italy (Silvestroni and Bianco, 1952), in America (Powell, Rodarte, and Neel, 1950; Sturgeon, Itano, and Valentine, 1952; Neel, Itano, and Lawrence, 1953), and in Greece (Caminopetros, 1952). The inheritor of both traits suffers as a rule from haemolytic disease of moderate severity. The following family appears to be the first described in England. It was discovered by the coincidental illness of one of its members.

* Two further haemoglobins have been recently described, haemoglobin E (Itano, H., Bergren, W. R., and Sturgeon, P., 1954, *J. Amer. chem. Soc.*, **76**, 2278), and haemoglobin G (Edington, G. M., and Lehmann, H., 1954, *Lancet*, **2**, 173).

Case Report

Mary H., aged 12 years 5 months, was sent to hospital on February 20, 1951, at 1 a.m. with a two-day history of sore throat, abdominal pains, and nausea. She was a thin, intelligent child, her facies definitely Caucasian (Fig. 1). The tonsils were red and the fauces injected, temperature 100° F. (38.4° C.), pulse 120 per minute. There was tenderness and " guarding " in the right lower quadrant of the abdomen and she was markedly tender on the right side per rectum. The liver and spleen were not palpable. A white cell count was 14,500 per c.mm. (neutrophil polymorphs 82%, 11,890, lymphocytes 12%, 1,740, monocytes 6%, 870. A diagnosis of acute appendicitis was made and appendicectomy was performed at 3 a.m. The appendix was thickened, tortuous, reddened, and inflamed. On the morning of February 22 the patient was definitely jaundiced. There was no bilirubin in the urine. The liver and spleen were palpable two fingerbreadths below the costal margin. The blood was examined and showed haemoglobin 7.55 g./100 ml., red blood cells 2,700,000 c.mm., packed cell volume 26%, M.C.V. 95 μ^3, M.C.H. 27.5 $\mu\mu$g., M.C.H.C. 29.0%. The film showed that practically all the cells were target cells (Fig. 2), with much punctate basophilia and 17% of reticulocytes. The red cell fragility was very much decreased. Haemolysis began at 0.32 NaCl and was complete at 0.16 NaCl. The patient made a good immediate recovery, but suffered a second haemolytic crisis one month later associated with the symptoms of pulmonary infarct. A further haemolytic crisis also connected with a throat infection occurred on June 14, 1951. Since that date she has been very well. The liver and spleen were only palpable during the crises. A radiograph of the skull and long bones showed no abnormalities.

Family History

The mother comes from Naples where her family have always lived. The father is the son of a West African woman and a white male (nationality unknown). The patient has three brothers (Fig. 1) who are well and without anaemia. Preliminary estimations were performed upon the blood of the family, namely, haemoglobin estimation, red cell count, examination of stained films, Price-Jones curves (Humble and Belyavin, 1948), osmotic saline fragility, reticulocyte count, and tests for sickling, in particular the 2% sodium metabisulphite test (Daland and Castle, 1948). In addition, the blood of the mother's mother, and of the mother's brother were kindly examined for us in Naples by Dr. Maurea, who has given us permission to include his results. In order to exclude any irregularity in the family their blood groups were examined in the ABO, M, N, S, P, CDE/cde, Kell, Lewis, and Duffy group systems by Dr. Mourant of the Lister Institute, who has been good enough to allow us to reproduce his results in this paper These preliminary results are summarized in Tables 1 and 2 and Figs. 3 and 4. They suggest that Mr. H. is a sickle-trait carrier, Mrs. H. is a mild case of Mediterranean anaemia, H. H. and L. H. (brothers) are sickle-trait carriers and have very mild Mediterranean anaemia, C. H. (brother) is normal, M. H. is a sickle-trait carrier (Fig. 5) with moderately severe Mediterranean anaemia?, Mrs. M. (grandmother) a slight degree of Mediterranean anaemia?, Mr. C. (mother's brother) normal. Three further investigations were then performed, namely (1) tests for foetal haemoglobin, (2) solubility of reduced haemoglobin specimens in buffers of high ionic strengths, (3) electrophoresis on paper of the carboxyhaemoglobins of the family's red cells.

FIG. 1.—The H family. At back (left to right) M. H., Mrs. H., and H. H. In the front C. H. and L. H.

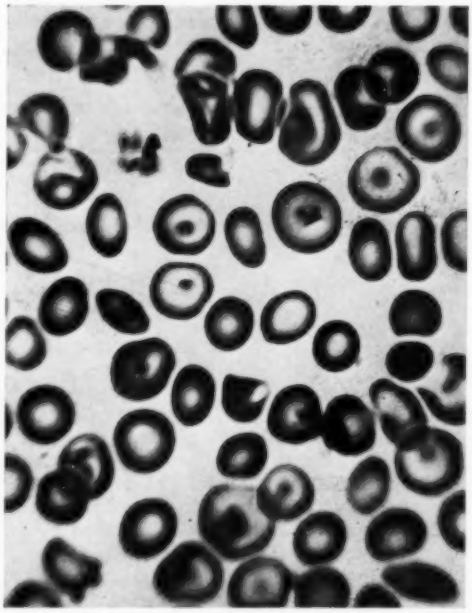

FIG. 2.—Blood film of M. H. to show target cells. × 1050.

TABLE I
HAEMATOLOGICAL FINDINGS IN THE "H" FAMILY

	Mr. H.	Mrs. H.	Mary H.	Hector H.	Leonard H.	Charles H.	Mrs. M. (Grand-mother)	Mr. C. (Maternal Uncle)
Haemoglobin (g. per 100 ml.)	18·06	13·32	7·55	12·88	13·32	13·47	12·73	15·09
Red cells (10^6 c.mm.) ..	—	5·02	2·74	4·55	5·02	4·5	6·11	6·26
P.C.V.	—	42	26	38·5	38	40	—	—
M.C.H.	—	26·4	27·55	28·31	26·4	30	—	—
M.C.H. (%)	—	31·71	29 03	33·1	35	33·67	—	—
M.C.V.	—	83·66	94·89	85	75·7	88·88	—	—
M.C.D.	7·22	7·36	7·73	7·51	7·41	7·36	—	—
S.	0·41	0·559	0·686	0·494	0·49	0·4	—	—
M.C.A.T.	—	1·96	2·02	1·92	1·76	2·01	—	—
D/T ratio	—	3·74	3·82	3·91	4·2	3·5	—	—
M.C.F. (NaCl g./100 ml.)	0·425	0·385	0·325	0·375	0·415	0·46	0·4	0·38
Reticulocytes	1	1·2	17·8	1·4	0·2	0·8	—	—
Sickling	Positive	Negative	Positive	Positive	Positive	Negative	Negative	Negative
Target cells	Nil	Many	Many	Few	Occasional	Nil	Nil	Nil

TABLE II
BLOOD GROUPS OF THE "H" FAMILY

Name	ABO	Rh	MN	S	P	Fy_a	Le^a	Le^b	Kell
Mr H. ..	O	rr cde.cde	M	–	+	–	–	–	–
Mrs. H. ..	B	R_1r CDe.cde	M	+	+++	+	–	–	–
M. H. ..	B	rr cde.cde	M	+	O	–	–	–	–
H. H. ..	O	rr cde.cde	M	–	+	–	–	–	–
L. H. ..	O	rr cde.cde	M	+	++	–	–	–	–
C. H. ..	O	rr cde.cde	M	–	+++	–	–	–	–

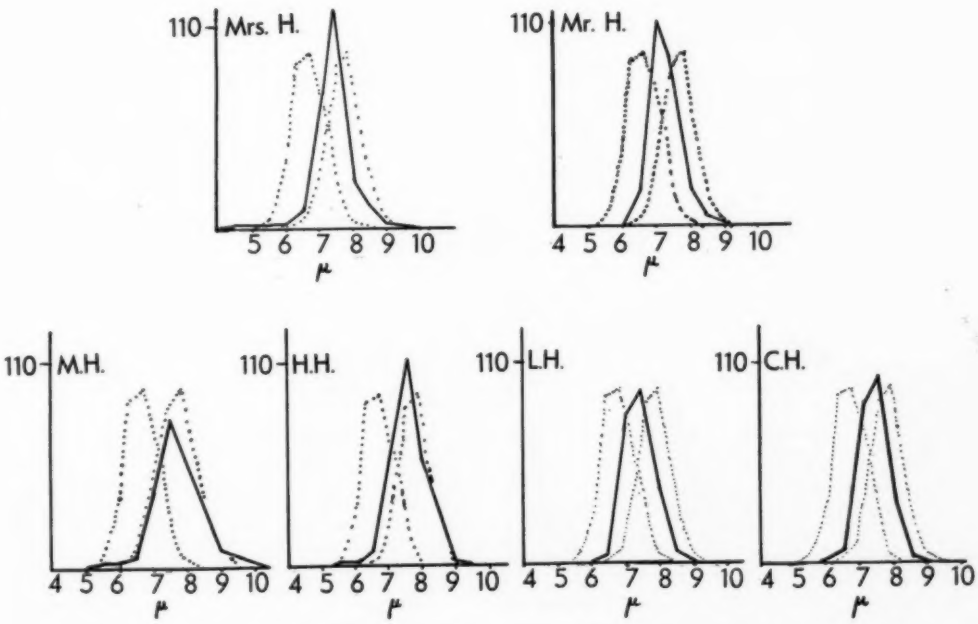

FIG. 3.—The Price-Jones curves of the H family, to show the "broad-based" curves of Mrs. H., M. H., H. H., and L. H.

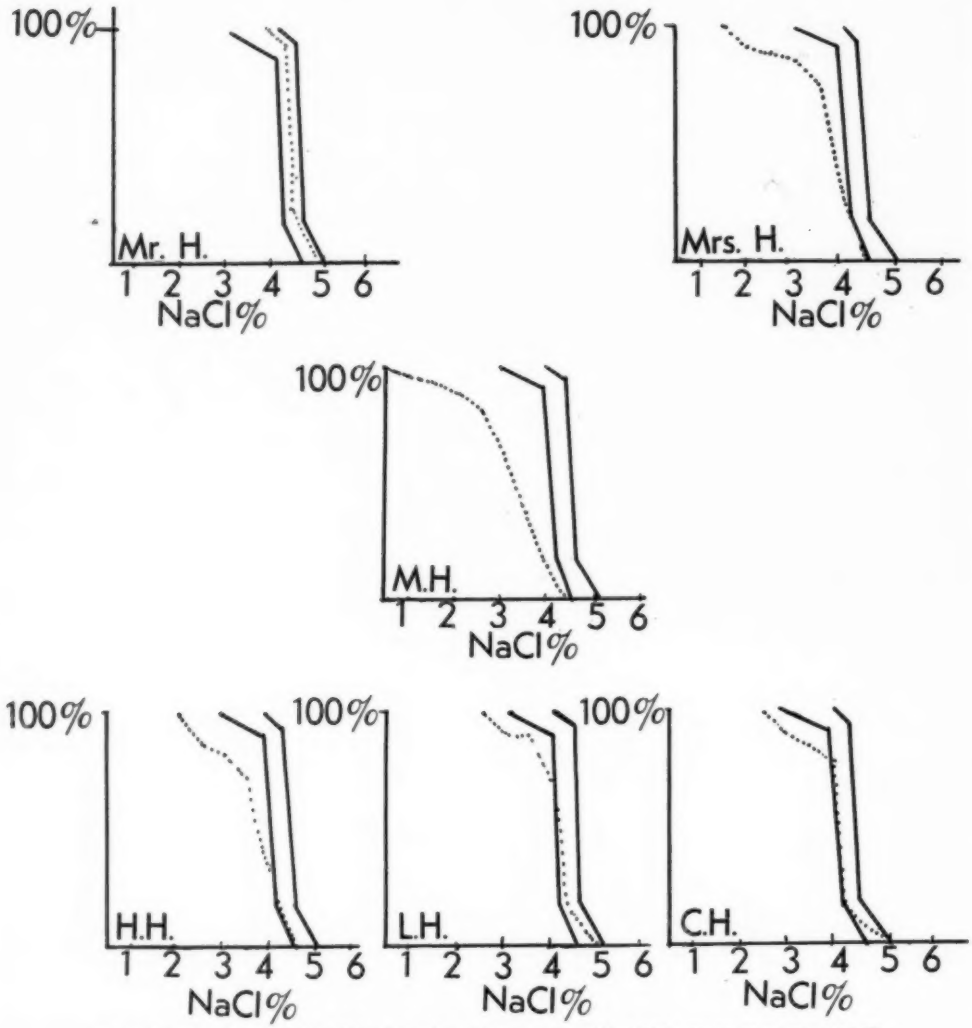

FIG. 4.—The saline fragilities of the H family to show the decreased fragilities of Mrs. H., M. H., H. H., and L. H.

Test for Foetal Haemoglobin.—No foetal haemoglobin could be detected in any member of the H. family using the method of alkali denaturation and two ultra-violet spectrophotometric methods (Beaven and White, 1953). These tests were carried out by one of us (J. C. W.) as part of a series to be reported elsewhere in cooperation with the M.R.C. Spectrographic Unit, London Hospital, with Drs. G. H. Beaven and E. A. Johnson.

Determination of Solubility of Reduced Haemoglobin in Phosphate Buffer pH 6.7 of High Ionic Strength.—Solubilities of reduced haemoglobin samples were determined by salting-out in *p*H 6.7 phosphate buffer at four high ionic strengths, r/2, 3.84, 4.31, 4.75, and 5.45. In this range, both normal and sickle-cell haemoglobins obey the linear Cohn relationship between log solubility and ionic strength (Cohn and Edsall, 1943; Jope and O'Brien, 1949; Perutz and Mitchison, 1950; Perutz, Liquori, and Eirich, 1951).

The tests were set up in 12×100 mm. pyrex tubes with the requisite amounts of stock 3.2M buffer of *p*H 6.7 (Green, 1933) and water, concentrated destromatized haemoglobin solution from laked erythrocytes, and

FIG. 5.—" Moist stasis " preparation of the blood of M. H. to show sickle cells. × 1050.

reducing agent to a total volume of 5 ml. The reducing agent was 0.1 ml. of freshly prepared M/2 ferrous citrate (Jope and O'Brien, 1949). The setting up was performed with a stream of nitrogen playing over the tube contents, and after sealing with a rubber stopper and a coating of paraffin wax-vaseline compound, equilibration was carried out for five days at 22° C., with gentle mixing on each of the first three days. The tubes were opened in the presence of nitrogen and the clear liquid phase pipetted off, or if necessary, centrifuged first under liquid paraffin. The solution was then treated with carbon monoxide, and the carboxyhaemoglobin estimated in the photoelectric colorimeter against the Haldane standard, 100%—14.8 g./100 ml.

The results are expressed in Fig. 6, where the heavy line 1 is drawn through the mean points at the ionic strength for the solubility of reduced haemoglobin of 13 individuals with normal blood or with various non-sickling anaemias. Line 3 similarly represents the mean solubility of four sickle-cell anaemia haemoglobin samples, and line 2 the mean solubility of 11 sickle-cell trait samples. The results on the haemoglobins of the family show that the son, C. H., possesses haemoglobin of normal solubility and that the haemoglobins of the father, the sons H. H. and L. H., and the patient M. H. possess solubilities comparable with those found in other sickle-trait carriers. It will be seen that then the solubility of the mother's haemoglobin is on the low side of normal; a trace of sickle haemoglobin cannot be excluded unequivocally by this method.

Electrophoresis on Paper of Carbon Monoxide Haemoglobins.—Samples of blood were collected in oxalate; the red cells were washed five times in normal saline, the final suspension of cells in saline was left overnight at 3° C. in an atmosphere of carbon monoxide. Finally, the saline was replaced by distilled water saturated with carbon monoxide and stored at —5° C. After storage the haemoglobin content of the samples was adjusted to 3 g.%, centrifuged at 6,000 r.p.m. for an hour; to the supernatant was added 0.5 g.% of pure albumin; this mixture was used for electrophoresis. (Albumin has a much higher mobility than haemoglobin, and serves to increase the separation of the haemoglobins.)

Electrophoresis.—Carboxyhaemoglobin, 20 mg., was put on Whatman No. 1 filter paper and placed in a modified partition box (Flynn and de Mayo, 1951). Carbon monoxide was passed through the box for three nours followed by nitrogen (oxygen free); then while

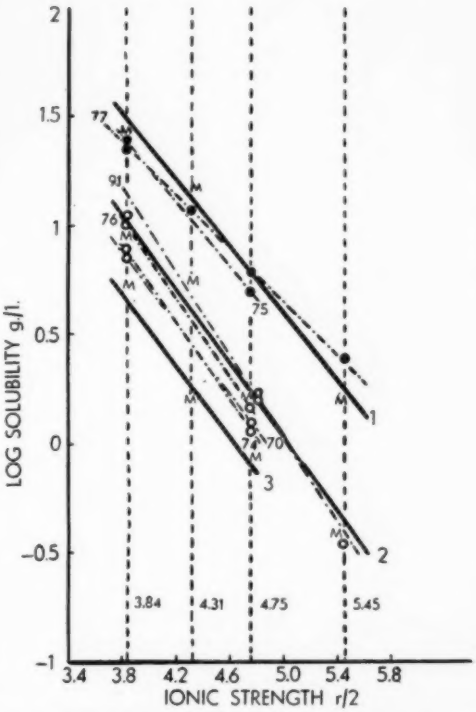

FIG. 6.—Solubility of reduced haemoglobin in *p*H 6.7 phosphate buffer at various ionic strengths. Line 1. Mean solubility of 13 normal haemoglobins. Line 2. Ditto for 11 sickle-cell trait haemoglobin specimens. Line 3. Ditto for four sickle cell anaemia haemoglobin specimens. 91 is Mr. H., 75 is Mrs. H., 70 is M. H., 74 is H. H., 76 is L. H., 77 is C. H.

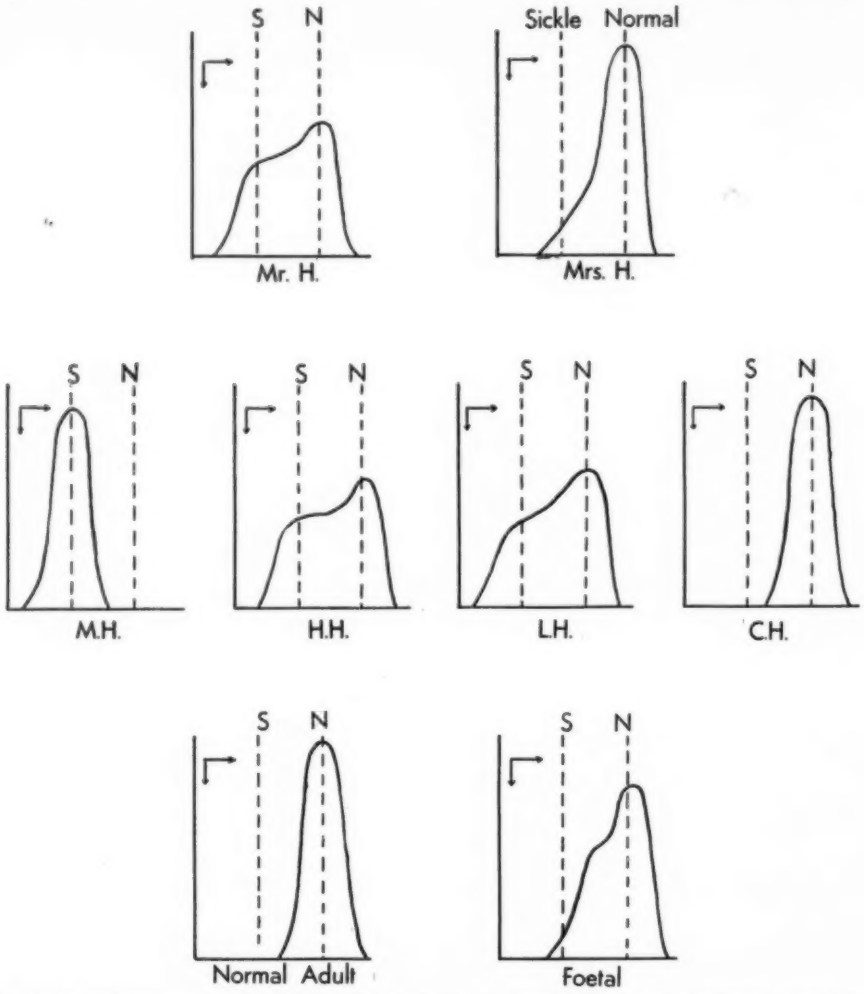

FIG. 7.—The electrophoretograms of haemoglobin samples from the H family together with a normal specimen and a specimen of cord blood. Paper-electrophoresis, buffer *p*H 8.6.

the nitrogen flow continued the troughs were filled with barbiturate buffer at *p*H 8.5. Finally, when the paper was saturated with buffer, a current of 350 v. 1.0 ma/cm. width was applied and allowed to run for 48 hours. The whole procedure, after the application of the haemoglobin solutions, being carried out with the exclusion of light to prevent a shift to the right in the reaction $CO\,Hb \rightleftharpoons C\,O + HbO_2$.

Estimations were made, after staining with a purified naphthalene black, with a mechanical scanner automatically recording on light-sensitive paper, using a scale calibrated by known quantities of haemoglobin solution (Crook, Harris, and Warren, 1952). The photo recordings were plotted on a linear scale; the area under each peak then corresponds to the amount of protein present.

Results.—Fig. 7 shows the electrophoretic pattern of normal adult and foetal carboxyhaemoglobin compared with the patterns of the H. family. It will be seen that at *p*H 8.5 normal haemoglobin moves faster than foetal or sickle haemoglobin. Mr. H. shows a mixture of sickle and normal haemoglobin; Mrs. H. shows a majority of normal haemoglobin, but there is some tailing

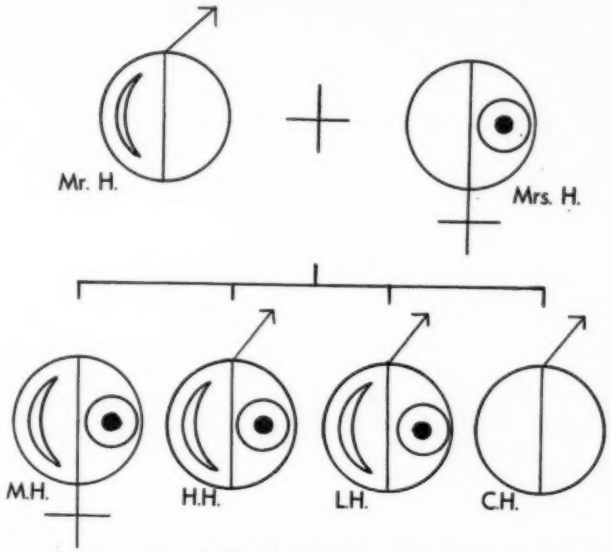

FIG. 8.—The inheritance of sickle trait and Mediterranean anaemia in the H family. ◖ = sickle trait. ○ = Mediterranean trait.

on the paper. Of the children, M. H. shows a predominance of sickle haemoglobin by this technique; H. H. and L. H. show a mixture of sickle and normal haemoglobin, and C. H. shows entirely normal haemoglobin.

Further electrophoretic experiments were made by adding the dialysed fractions obtained in the solubility experiments to known normal haemoglobin. It was found that 4.5% of sickle haemoglobin could not be detected in the presence of 95.5% adult haemoglobin. Conversely, 4.5% of normal naemoglobin could not be detected in the presence of 95.5% sickle haemoglobin. It is thus impossible to categorize with certainty the trace of ? abnormal haemoglobin seen in the electrophoretogram of Mrs. H. or the trace of ? normal haemoglobin in that of M. H.

These special methods confirmed the opinion produced by the conventional examinations that there were two conditions present in this family. The inheritance of the two conditions is set out in Fig. 8.

Discussion

Clinically these cases are of great interest, and two differing syndromes are often mistaken for the haemolytic crises which so frequently occur. As they suffer from fever, pain in the bones, and a loud heart murmur, a diagnosis of rheumatic fever is frequently made. Our patient was so diagnosed at the age of 6. The second common syndrome is an attack of pulmonary thrombosis causing an infarct, again a feature of our patient's history. It will be noted that the patient M. H. is anaemic and has a much more abnormal fragility curve, and an abnormally flattened Price-Jones curve compared with her two affected brothers. These results suggest that if the sickle trait is associated with an inheritance of Mediterranean anaemia, then the two conditions augment each other and produce clinical symptoms (Silverstroni and Bianco, 1952). It will be seen that the patient M. H. has much more sickle haemoglobin than her brothers (Fig. 7). This factor also would tend to make symptoms more likely. Although the mother's haemoglobin is predominantly of the normal and adult form, the examination does not completely preclude the co-existence of a small percentage of sickle haemoglobin. This rare possibility has been described by Singer and Fisher (1953), but the numerous target cells, slight hypochromia, and increased resistance to saline haemolysis of the red cells are consistent with the occurrence of Mediterranean trait in this southern Italian woman (Wintrobe, Matthews, Pollack, and Dobyns, 1940). It appears that the double inheritance of the sickle cell Mediterranean anaemia is responsible for many of the cases reported in the literature of sickle cell anaemia occurring in white races. The association of sickling with the Negro races has produced in this connexion many circuitous and unnecessary explanations, for it is clear from the study of this family that the sickle-trait may lie concealed beneath the Caucasian features.

Summary and Conclusions

A family exhibiting the double inheritance of the sickle-trait and Mediterranean anaemia is described.

The family was discovered by the coincidental illness of one of its members.

The presence of sickle-cell haemoglobin was demonstrated in the blood of the father, of the affected daughter, and of two of the sons by determining the solubility of the haemoglobin in the reduced state.

Sickle-cell haemoglobin was also demonstrated in the blood of these four members of the family by the technique of paper electrophoresis, but not in the blood of the mother or of the third son.

Attention is drawn to the additive effect of the two conditions in the production of clinical disease.

The authors wish to acknowledge with gratitude the assistance given by Dr. Mourant in determining the blood groups of the family and to Dr. Maurea for examining the blood of Mrs. M. and Mr. C. Our thanks are also due to Dr. Peter Hansell, of the Department of Medical Photography, Westminster Medical School, for his assistance with the illustrations.

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ALKALI-RESISTANT HAEMOGLOBIN IN NORMAL GREEK CHILDREN

BY

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The time of disappearance of embryonic haemoglobin in post-natal life has not yet been definitely determined.

Since Körber in 1866, Krüger in 1888, and later Bischoff (1926) and Haurowitz (1929, 1930) demonstrated the existence of a difference between the resistance of foetal and adult haemoglobin to the denaturing action of alkali, the former being much more resistant than the latter, few papers on this subject have appeared in the literature.

Using electrophoretic and spectroscopic methods Beaven, Hoch, and Holiday (1951) demonstrated that the substitution of foetal haemoglobin by the adult pigment starts during intra-uterine life. The youngest embryo examined was 20 weeks old, and 6% of its haemoglobin was found to be of the adult type. These authors found that at birth the adult pigment usually amounts to about 20%, which agrees with the previous findings of Haurowitz, while between the third and fourth month about 90% of foetal haemoglobin has been replaced by adult haemoglobin. This is easily explained by the fact that embryonic red cells are still in the blood stream, the life span of erythrocytes having been estimated to be 120 days. Yet embryonic pigment is still present in small amounts beyond this age, a fact which suggests its post-natal production. The time limit for its disappearance was believed until recently to be the seventh month of life.

Chernoff and Singer (1952) demonstrated that foetal haemoglobin could be found up to the fifth year of life in amounts of 3.1%. This discrepancy with the older literature Singer attributed to his more sensitive method (Singer, Chernoff, and Singer 1951). Instead of measuring the time needed for denaturation of haemoglobin with alkali, Singer determined the percentage of haemoglobin denatured within one minute and stated that very small amounts of resistant pigment—up to 1.7%—can still be demonstrated in normal adults.

Greater amounts of foetal haemoglobin are encountered as a rule, and independently of age, in sickle cell anaemia and in Cooley's anaemia (Singer *et al.*, 1951; Bianco, 1948; Zannos, 1952). As these diseases are frequent in Greece, we thought it would be interesting to find the age limits at which foetal haemoglobin has completely disappeared from the blood of Greek children. The problem is also of practical interest, since the method of haemoglobin alkali denaturation serves as a diagnostic method for distinguishing the congenital from acquired types of anaemia.

Material and Method

The present paper deals with the determination of alkali-resistant haemoglobin in 500 Greek children aged between 2 and 7 years. Three hundred and fifty of these children were patients at the paediatric clinic of Athens University, and suffered from diseases which do not affect the haemopoietic system. The remaining were healthy nursery and school children.

Singer's (1951) method was used.

Results and Discussion

As shown in Table I, foetal haemoglobin is still present in a very small percentage of children up to the seventh year of life. The amount is relatively high during the third year, 1.9=6.9%, decreasing gradually to 2=2.6% during the seventh year. Investigation for blood disease or sickle cell trait or thalassaemia in the cases with foetal pigment yielded negative results.

TABLE I

PERCENTAGE OF FOETAL HAEMOGLOBIN AT DIFFERENT AGES

Age in Years	No. of Specimens Examined	No. of Specimens with Foetal Hb	Percentage Foetal Hb
2–3	100	23	2–6·9
3–4	100	11	2–5·5
4–5	100	10	2–5·1
5–6	100	8	2–4·5
6–7	100	2	2–2·6

Comparison of our results with those of Chernoff and Singer shows a slight difference in the age limit of disappearance (seventh instead of the fifth year) as well as in the percentage of foetal haemoglobin, but we do not think that this difference is statistically significant, given the relatively small number of Singer's cases.

It is hoped that the present investigation will stimulate similar studies in other parts of the world, so that further comparisons would be made possible.

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MYELOSIS INVOLVING THE GRANULOCYTIC AND ERYTHROCYTIC SYSTEMS

BY

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(RECEIVED FOR PUBLICATION DECEMBER 11, 1953)

There are several syndromes in which an erythroblastic reaction, comparable with the granulocytic reaction of granulocytic leukaemia, is prominent. One is the "leukanaemia" of von Leube (1900) defined by Foy, Kondi, and Murray (1946) as "a haematological condition in which there co-exist a pernicious anaemia-like and a leukaemia-like blood picture with corresponding marrow disorder of the erythroid and myeloid issues"; they conclude that "as a disease *sui generis* [it] does not exist, being either atypical pernicious anaemia or atypical myeloid (usually myeloblastic) leukaemia." Garnier, Cordier, and Sigwalt (1941) described a variant unusual in its intensity, and many other variants may be found in the French and Italian literature. Anglo-Saxon writers rarely describe such cases, for they usually regard the erythroblastosis or the granulocytic reaction, as the case may be, as symptomatic epiphenomena of little importance, and "erythroleukaemia" and "leukanaemia" are dismissed each in 15 to 20 lines by Whitby and Britton (1950).

However, Di Guglielmo (1945) has brought forward evidence which suggests that the acute and chronic erythraemic myeloses which bear his name may well prove to be disease entities; he also describes an erythroleukaemia which can be difficult to differentiate from erythraemic myelosis on the one hand and leukanaemia on the other. Acute erythraemic myelosis, always fatal within two months of onset (Di Guglielmo, 1923, 1926, 1945), is characterized by anaemia, fever, splenomegaly proportionally greater than hepatomegaly, and proliferation in marrow, spleen, lymph nodes, and liver of dysplastic erythroblasts which fail to mature. The nuclei of these cells are usually megaloblastoid or reticuloendothelioid. The chronic form (Duesberg, 1940; Heilmeyer and Schöner, 1941; Di Guglielmo and Quattrin, 1942) has a more chronic course, up to two years, and the peripheral blood erythroblasts are orthochromatic. It is the only form so far described in England (Israëls, 1939; Neumark, 1949; Emery, 1951). In erythroleukaemia hepatomegaly may be more marked than splenomegaly, and erythroblastosis, though it persists till death, is more intense at the beginning of the disease, giving place to the uncontrolled proliferation of primitive granulocytes which may have grossly atypical, histiocytic nuclei.

Recently, evidence has been accumulating that erythraemic myelosis and erythroleukaemia may be unusual modes of onset of acute leukaemia (Bussi and Franzini, 1951; Rachet, Mallarmé, and Bourel, 1951; Mallarmé and Bourel, 1951). It appears that the longer the patient survives, the more likely is he to develop the classical picture of acute granulocytic leukaemia, and now that intensive transfusion is so commonly practised patients with leukaemia-like syndromes rarely die from anaemia in the early stages of the disease, so that the change from erythraemic myelosis to leukaemia is more commonly seen. Nevertheless, patients still die with the picture of erythraemic myelosis (Emery, 1951; Schwartz and Critchlow, 1952). It seems as yet uncertain whether erythroleukaemia will have to be separated from erythraemic myelosis, or whether these "diseases" can be grouped together as unusual variants of acute granulocytic leukaemia. The case recorded here appeared at first to be erythraemic myelosis, but ultimately had to be classed as erythroleukaemia, resembling closely the case of Verloop, Deenstra, and van der Hoeven (1952).

Case History (No. 10272/52)

A man aged 72, who had previously never been ill, noticed lassitude and dyspnoea from January, 1952, became increasingly weak, short of breath, and developed pain in the chest on exertion from March, 1952, and collapsed in the street about the end of April. He refused to enter hospital. He did not improve and his weakness became so extreme that he was scarcely able to leave his bed. On admission on June 3, 1952, he was very pale, had a black tongue, enlarged cervical and axillary lymph nodes, and oedema of both ankles. The liver was palpable 3 in. (8 cm.) below the costal margin,

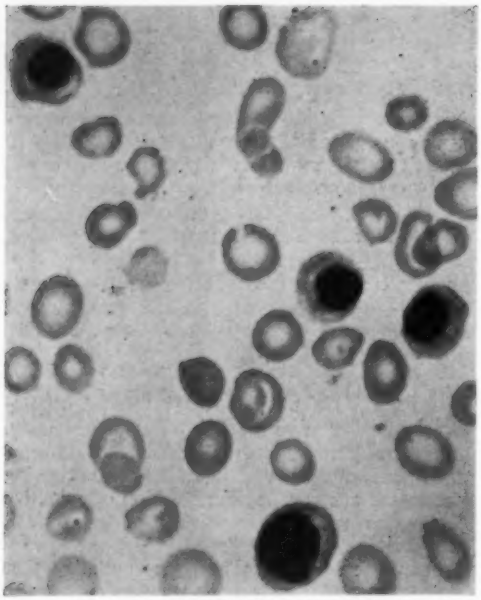

FIG. 1.—Film of peripheral blood on admission.

but the spleen could not be palpated. The apex beat was $\frac{1}{2}$ in. (1.5 cm.) outside the mid-clavicular line, and a gallop rhythm was present, though no abnormal sounds were heard (pulse 96, blood pressure 182/90 mm. Hg). The blood count (Hb 4.7 g., nucleated red cells 26,200 per c.mm.) and sternal marrow were considered diagnostic of a subacute erythraemic myelosis (Tables I and II).

The course was progressively downhill in spite of transfusion. On June 10 he had ecchymoses of the hard palate and a petechial rash on the face. Blood oozed from every prick, in spite of a platelet count of 230,000 per c.mm. Low-grade fever up to 99° F. developed. Slight improvement occurred during the next week, but on June 18 he had an epileptiform attack in which a tooth was knocked out ; in spite of stitching the socket,

TABLE I

PERIPHERAL BLOOD

Cell Type	June 3	June 21	July 8	July 14	July 22
Leucocytes ..	2,100	745	2,550	2,440	10,000
Myeloblasts ..	850	100	100	—	1,380
Erythroblasts ..	26,200	4,500	215	428	5,570
% of normoblasts:					
Pro-	4·5	2·5	—	—	—
Basophilic ..	7·5	8·5	—	21·5	17
Polychromatic ..	35	15·5	—	42·5	38·5
Orthochromatic	53	73·5	100	36	44·5

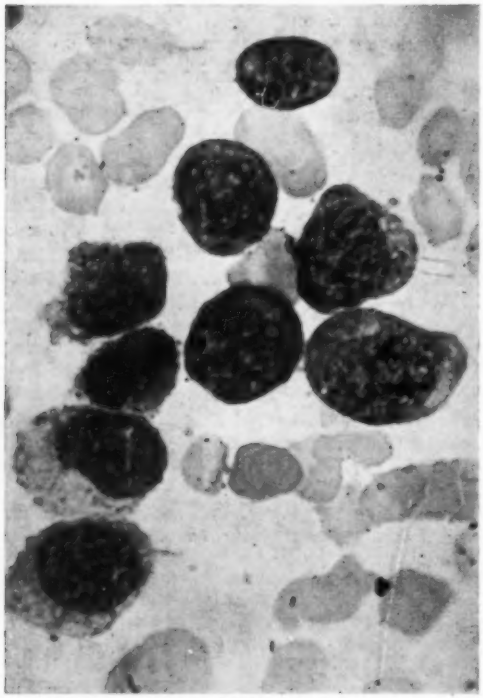

FIG. 2a.

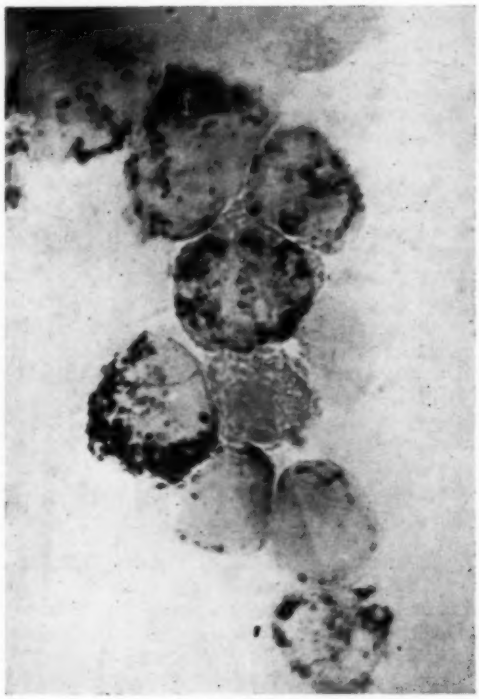

FIG. 2b.

FIG. 2.—Marrow films taken on day of death: (*a*) Leishman, (*b*) peroxidase stain. Nearly all cells contain granules.

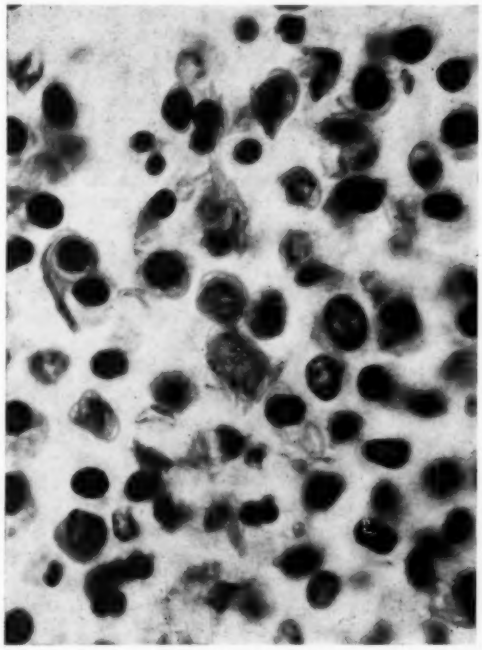

FIG. 3a.

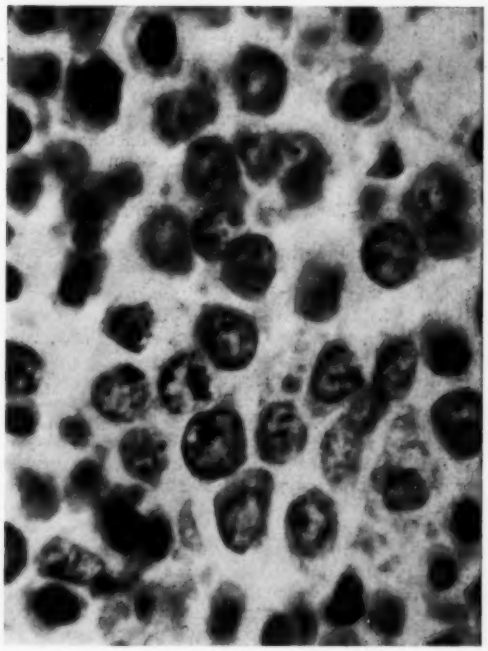

FIG. 3b.

FIG. 3.—Necropsy material. (*a*) and (*b*) sections of bone marrow.

TABLE II
MARROW DIFFERENTIAL COUNTS

Cell Type	June 4	July 22
	%	%
Haemohistioblast	—	5
Myeloblast	0·5	13
Progranulocyte	9·5	26
Neutrophil myelocyte ..	5·0	24·5
Juvenile	2·5	5·5
Staff form ..	0·5	3·0
Segmented ..	2·0	2·0
Lymphocyte	2·0	1·5
Plasma cell	0·5	0·5
Normoblasts:		
Pro-	6·5	2·0
Basophilic	16·5	5·5
Polychromatic	31·5	9·0
Orthochromatic	16·5	1·5
Mitotic	1·0	Nil
Naked nuclei	5·0	0·5
Unclassified	0·5	0·5

blood oozed continuously from it until death. On June 25 he had a melaena stool. On July 3 he was transfused up to 13.0 g. Hb (88%) with symptomatic improvement, but on July 8 he developed haematuria and a sub-conjunctival haemorrhage; intermittent bleeding from these sites continued until death on July 22, about seven months after the clinical onset, he having received 13.5 litres of stored blood during the last 50 days of life. His sister had died of monocytic leukaemia in Hammersmith Hospital in 1943.

Haematology.—The data are summarized in the tables and Figs. 1–3. The patient was admitted with anaemia, a reticulocytosis of 31%, and erythroblastaemia, the erythroblasts being mainly orthochromatic and showing karyorrhexis and a few mitotic figures. On June 10 the platelet count was 230,000 per c.mm. Leucocyte and erythroblast counts gradually fell until on June 8 primitive cells of either series were scanty; thereafter both increased and primitive cells became abundant, most clearly seen in the marrow of July 22 (vide infra).

Necropsy.—Necropsy showed petechial haemorrhages in the skin, pia mater, pericardium, endocardium, parietal and visceral peritoneum and pleura, gastro-intestinal tract, renal pelvis, and ureter.

The bone marrow was red and hyperplastic (sternum, ribs, femur, and vertebrae).

Lymph nodes of the cervical, axillary, para-aortic, mediastinal, pelvic, and inguinal groups were enlarged to about 1 cm. diameter: they remained discrete, were soft, and the cut surface was red.

The liver (1,930 g.) was pale and rather greasy in appearance; the normal architecture was preserved.

The spleen (217 g.) was soft, pink, and patchily congested.

The kidneys (L. 155 g., R. 135 g.) showed narrowed cortex and pale, mottled, cut surfaces.

The heart (470 g.) showed a rather fatty myocardium, the left ventricle being dilated and hypertrophied (wall 2 cm. thick). The coronary arteries showed little atheroma except 3 cm. from the ostium of the right coronary artery, where there had been subintimal bleeding at an atheromatous plaque. Other organs showed no gross abnormality.

Histology.—The myocardium shows some degenerative pigment.

The tongue and pituitary show no significant abnormality.

A kidney shows patchy leukaemic infiltration in cortex and medulla, and the suprarenals show granular deposits of pigment which does not contain iron, and a few foci of leukaemic cells.

The portal tracts of the liver are infiltrated by leukaemic cells; the parenchymal cells are swollen, and many contain droplets of bile pigment and granules of haemosiderin. The sinusoids contain leukaemic cells.

The bone marrow is extremely cellular, and very few typical myeloid or erythroid cells are present. A few reticulum cells are present, but the predominating cell is a rounded or polyhedral cell forming dense sheets. It has a copious, very finely granular, eosinophilic cytoplasm, with a central dense hyperchromatic nucleus, sometimes so dense as to suggest pyknosis. Some nuclei, however, are less dense and more vesicular, and these have usually less intensely eosinophilic cytoplasm. There are no cells with the typical morphology of myeloblasts.

The lymph nodes show (1) diffuse hyperplasia of cells resembling those of the bone marrow, which also infiltrate the capsule, and (2) areas of necrosis, among which are "follicles" made up of cells resembling those of the bone marrow. Staining of frozen sections by a benzidine blue method shows that the "follicles" are surrounded by cells containing many lipoid granules, which cells are also found in and around the necrotic areas: these cells are obviously not polymorphs but appear to be myelocytes or histiocytes.

In the spleen the Malpighian bodies are small and inconspicuous, separated by a slightly congested pulp. Reticulum cells are scanty, histiocytes are more numerous, but the sinusoids are filled with cells resembling those of the bone marrow, and islands of cells resembling typical normoblasts are present. There is no erythrophagocytosis, but much haemosiderin is present.

Discussion

Great difficulty was encountered in analysing the cytology of the bone marrow from sections, even though these were cut from material aspirated only half an hour after death. The predominating cell, described above, resembled no cell hitherto seen in the acute laekaemias, but bore somewhat greater resemblance to an erythroblast showing arrested maturation at the early polychromatic stage. Study of smears of the same marrow, stained by Leishman's stain and Sato and Sekiya's peroxidase reaction, led to quite different conclusions—that about 75% of the marrow cells were of the granulocytic series, promyelocytes, or myelocytes A and B on Sabin's classification.

At first inspection the predominating nuclear pattern of cells in the marrow film resembles most closely a megaloblast, with rather coarse reticulation, at the nodes of which occur large, apparently solid lumps of chromatin. However, some of these cells, even the most basophilic ones whose nuclei contain obvious nucleoli, contain also azurophil cytoplasmic granules, which are extremely rare in megaloblasts; and, in the peroxidase-stained film, approximately 75% of the cells were peroxidase positive. More critical examination indicated that the "megaloblastic" or "reticulo-endothelioid" appearance of the nuclei was due to two factors: (1) the presence of unusually numerous heterochromatic bodies (which are more conspicuous in megaloblasts than in other cells), and (2) the presence of many granules in the cytoplasm — large compared with mitochondria and some azurophilic. These granules, together with the mitochondria, underlie and overlie the nucleus and indent it, producing the stippled pattern normally seen in "blasts" (Jones, 1948); because of their unusually large size the stippling is unusually coarse, even coarser than is usually seen in megaloblasts and comparable with that seen in haemohistioblasts and reticulum cells.

It was surprising to note that few cells were intensely basophilic in spite of the active proliferation which appeared to be taking place.

It becomes ever more obvious that haemocytological studies are reliable only when based on smears or imprints of material obtained during life.

Conclusion

If we accept the validity of describing disease entities of "leukanaemia," "erythroleukaemia," "acute erythraemic myelosis," our patient must be regarded as suffering from subacute erythroleukaemia. Many observers doubt whether these distinctions are valid, considering that these names describe modes of onset of acute granulocytic leukaemia between which no sharp distinction can be drawn, and that the appearance of erythroblasts should be regarded as an epiphenomenon whose chief importance is that it makes diagnosis more difficult (see Rodellec Du Porzic, 1938; Lüdin, 1950; Sarrailhé, 1951; Bussi and Franzini, 1951; Mallarmé and Bourel, 1951, for the development of this idea). Further analysis of this concept must await the recognition of more cases.

Summary

A man aged 72 developed anaemia and died about 12 weeks from the onset of symptoms.

The anaemia was characterized by intense erythroblastaemia and reticulocytosis, with marrow erythroblastosis, and a haemorrhagic state: a diagnosis of Di Guglielmo's erythraemic myelosis was accepted.

At death the patient was found to have changes characteristic of acute granulocytic leukaemia.

We wish to thank Dr. Richard Asher for permission to describe this patient, who was under his care.

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NON-REACTIVE TUBERCULOSIS

BY

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Non-reactive tuberculosis has been reported under many different names. It is called acute caseating tuberculosis by Rich (1944), while on the Continent it is most commonly called *Landouzyschekrankheit* and the Yersin type of tuberculosis. It may be defined as a fatal form of tuberculosis in which many organs contain miliary foci of necrosis surrounded by normal parenchymal cells. Typically cellular response is completely absent, but occasionally there is a poor response of the usual type. Very large numbers of tubercle bacilli are found in and around these lesions. Usually in life there are gross abnormalities of haemopoiesis, such as anaemia or polycythaemia, agranulocytosis or a leukaemoid change, and thrombocytopenia. Rarely, the histological picture of non-reactive tuberculosis is found in leukaemia and myelosclerosis.

It is proposed to review the literature of non-reactive tuberculosis, to describe eight unpublished cases, and to discuss the pathogenesis of this condition.

TABLE I

SIXTY-SIX REPORTED CASES

Clinical Diagnosis	No.
Agranulocytosis	22
Leukaemoid	7
Panmyelophthisis	5
Polycythaemia	2
Normal blood picture	6
No record of blood	5
Leukaemia	13
Myelosclerosis	5
Hodgkin's disease	1
Total	66

There are sixty-six cases in the literature which fulfil the criteria of non-reactive tuberculosis. Table I shows the number of cases subdivided according to various haematological changes. A pre-existing disease process may influence the course of the tuberculous infection; accordingly, the 19 cases with leukaemia, myelosclerosis, and Hodgkin's disease have been isolated in the table. Forty-seven cases remain in the main group.

Other cases are often quoted, but they will not be considered as they are unacceptable for the following reasons: Weichmann (1922), Róth (1929), Holzer (case 2, 1927), and Reiche quoted by Bálint (1925) give no histological description. The cases of Reilly and Bolin (1931) and Loeschcke (1932) were newborn infants and were probably cases of transplacental infection.

The salient features of the 47 cases in the main group are summarized in Table II.

TABLE II

SUMMARY OF CASES REVIEWED WITH NO INTERCURRENT DISEASE

Haematological Group		Agranulocytosis	Leukaemoid Change	Panmyelophthisis	Polycythaemia	Normal White Count	No Blood Count	Total*
No.	Males	13	3	4	2	3	2	27
	Females	8	1	1	0	2	3	15
	Total	22	7	5	2	6	5	47
Mean age		40	38	49	49	42	44	43
Duration of illness	Less than 4 weeks	11	1	1	1	3	2	19
	4 " 12 "	5	3	0	0	3	0	11
	More " 12 "	2	2	4	1	0	1	10
Haemorrhagic incidents		5	3	2	1	0	0	11
Anaemia	Present	3	6	5	0	1	0	15
	Absent	8	1	0	0	0	0	9
Necropsy:								
Old primary	Present	2	2	2	1	0	2	9
	Absent	8	1	0	0	0	0	9
Spleen	Enlarged	10	5	5	1	4	3	28
	Normal	2	0	0	0	0	0	2
Liver	Enlarged	4	3	3	0	3	2	15
	Normal	4	0	2	1	0	0	7
No naked-eye abnormality		3	0	0	0	1	0	4
Necrosis in marrow		9	1	2	0	1	0	13
Type of bacillus	Human	6	0	2	0	0	2	10
	Bovine	2	0	0	0	2	0	4

In places in the table the number of cases in some of the subdivisions does not add up to the total number of cases in the particular group. This is due to an absence of the relevant information in some of the papers.

Clinical Course of Reported Cases

The commonest course seems to have been an acute, overwhelming infection. Less often the syndrome presented as a chronic pyrexia of

unknown origin (Siegmund, 1939, case 6), sometimes as an obscure anaemia, or apparently as panmyelophthisis (Arends, 1950) or as primary agranulocytosis. The terminal phase of the syndrome may be complicated by tuberculous involvement of many organs. The skin sensitivity to tuberculin was investigated only three times. Twice it was weakly positive (Gibson, 1946; Arends, case 2, 1950), and once negative (Hegler, 1938).

Post-mortem Appearance

It is remarkable that the post-mortem examination in four cases revealed splenomegaly, but no other macroscopic change suggestive of tuberculosis. In the other 27 cases, in which lesions were described, they varied from pinhead size up to yellow areas of necrosis of 1 cm. in diameter. The spleen was nearly always reported as enlarged, while the liver was often enlarged. The glands often showed large necrotic or caseous areas (Pagel and Woolf, 1949; Smith, case 1, etc., 1949), but more usually they were normal. An old primary focus has been reported nine times, but several workers, particularly Siegmund (1939), have stressed its absence; he found no primary in seven of the nine cases in which a reasonably thorough search appears to have been made for a primary lesion. Lastly, there are several reports of tuberculous infection in a large number of organs. There is one notable exception to this list. No case of tuberculous meningitis has been reported.

Histology

The characteristic histological appearance was a small area of necrosis, sometimes with the "ghost" parenchymal architecture still just visible. Appropriately stained sections showed enormous numbers of acid-fast bacilli packed together forming felt-like masses. Surrounding this area were normal parenchymal cells, but no inflammatory or other foreign cells. This lesion is described in the majority of reports (for example, in the photomicrographs of Arends), but in quite a number of cases there was a mild degree of lymphocytic or monocytic cuffing (Gibson, 1946). Gibson (1946), Holzer (1927), and others have found evidence in some areas of a more or less normal tissue response with a lymphocytic cuff, epithelial cells, and young fibre formation. In all the cases accepted the atypical aspect of the body response has been stressed or clearly described, but in some cases there was also a response intermediate in form between the typical non-reactive lesion and that in the normal tissue.

The non-reactive lesions were always found in the liver and spleen. They were almost always present in the marrow. In the leukaemoid cases the marrow was usually very cellular; indeed, in these cases only the absence of leukaemic cellular infiltration in the spleen and liver made possible the exclusion of a diagnosis of leukaemia. Other reports (Marzullo and de Veer, 1931; Blair and Pagel, 1947; Custer and Crocker, 1932) have described a hypocellular and even fibrotic marrow. Commonly foci are found in the lungs and kidneys, but any organ, except, it seems, the meninges, may occasionally be similarly affected.

Bacteriology and Mycology

Coincidental mycelial infection was reported in four cases (Siegmund, case 3, 1939; Esser, 1926; Smith's cases 2 and 4, 1949). The type of tubercle bacillus was identified by adequate cultural methods and animal inoculations in 14 cases. It was found to be human in 10 cases and bovine in four.

Blood Changes

Agranulocytosis.—Of the 47 cases now under discussion, 22 had leukopenia, or a total absence of polymorphs (Leibowitz, 1938; Marzullo and de Veer, 1931; Smith, case 3). Anaemia was not a feature. Thrombocytopenia was present in the few cases in which the platelets were counted, and was almost always associated with purpura. When investigated, the marrow was usually hypoplastic.

Leukaemoid Count.—In the seven cases accepted as having a leukaemoid blood picture, the total white count varied from 200,000 (Custer and Crocker, case 1, 1932) to 4,000 (Gardner and Mettier, 1949; Leibowitz, 1938; Staffurth and Spencer, 1950), but all had a high proportion of blast cells or myelocytes (Custer and Crocker, case 1, 1932) or prolymphocytes (Staffurth and Spencer, 1950) or even monoblasts (Gibson, 1946). Clinically, these patients survived on average a little longer than those with agranulocytosis, and they had splenomegaly and anaemia and haemorrhagic incidents more frequently.

Panmyelophthisis.—Four cases included are under the heading of panmyelophthisis, all of which had thrombocytopenia, granulopenia, and anaemia, which was extreme in two. Haemorrhagic incidents were reported in two cases (Arends, case 1, 1950; Kernohan, 1950). Otherwise the clinical course in these few cases did not differ from that of the whole group.

Polycythaemia.—Two cases (Rennen, 1922; Lederer, 1923) were thought clinically to have polycythaemia. Both had over 7.5 m. red cells per c.mm. and one (Rennen) had a high normal white count, with 90% polymorphs and an occasional nucleated red cell.

Normal Blood Count.—In six cases the total white count was within normal limits, and the differential count when given was also normal. One of these cases had previously been diagnosed as having pernicious anaemia, but was having adequate treatment at the time of his final illness (von Wyss, case 5, 1940).

No Blood Count Reported.—Five cases are included in which the blood was not investigated, but which are acceptable since an adequate histological description was given.

Leukaemia.—In all the cases referred to so far the diagnosis of leukaemia could be excluded with reasonable certainty. Thirteen cases, most of which undoubtedly had leukaemia, will now be reviewed. A summary of the outstanding features in the cases of leukaemia, myelosclerosis, and Hodgkin's disease is presented in Table III. When the

TABLE III
SUMMARY OF CASES REVIEWED WITH INTERCURRENT DISEASE

Haematological Group		Leukaemia	Myelosclerosis	Hodgkin's Disease	Total
No.	Males	2	3	—	5
	Females	6	2	1	9
	Total	13	5	1	19
Duration of illness	Less than 4 weeks	1	—	1	2
	„ „ 12 „	2	—	—	2
	More „ 12 „	4	5	—	9
Haemorrhagic incidents		3	1	0	4
Anaemia	Present	3	5	—	8
	Absent	—	—	—	—
Necropsy:					
Old primary	Present	4	1	—	5
	Absent	1	—	—	1
Spleen	Enlarged	7	5	0	12
	Normal	2	—	1	3
Liver	Enlarged	3	5	0	8
	Normal	1	—	1	2
No naked-eye abnormality		1	—	0	1
Necrosis in marrow		—	3	1	4
Type of tubercle bacillus	Human	3	—	—	3
	Bovine	1	—	—	1

differential diagnosis between leukaemia and a leukaemoid change was in doubt, the cases have been included. Coley and Ewing's case (1911) probably had acute lymphatic leukaemia. Chronic myeloid leukaemia was present in seven cases, while lymphoblasts were found in one case (Fischer, 1935, case 1).

The course of the leukaemia seems to have been in no way abnormal, while that of the tuberculosis was usually rapid. In four out of five cases in which there was relevant information, there was evidence of old tuberculosis. The histological picture of the non-reactive tuberculosis was typical except that if there was any cellular response the cell type was that of the leukaemia.

Myelosclerosis.—There are two reports of this histological picture occurring in people dying with myelosclerosis (Carpenter and Flory, 1941; Crail, Alt, and Nadler, 1940). In each case the myelosclerosis had been present for many months. Crail *et al.* report four cases associated with non-reactive tuberculosis and contrasts these with five other cases of idiopathic myelosclerosis. His tuberculous patients were all comparatively young and all were febrile; the duration of the illness was shorter than that in the idiopathic group. He suggests that in these cases the tuberculosis first stimulates the fibrosis of the marrow and the glands and subsequent development of splenomegaly and the leuko-erythroblastic blood picture, with terminal haematogenous spread and the production of non-reactive lesions

Present Series

Case 1.—H. B., a man aged 33, complained of bleeding gums, a petechial rash, and haematuria. The only abnormal findings were some changes in the mid-zone of the left lung in the radiograph of the chest, a platelet count of 5,000 per c.mm., and a prolonged bleeding time. He was given a pint of fresh blood and improved clinically and the platelet count rose to 200,000 per c.mm. in 10 days. Forty-three days later he complained of wasting, vomiting, abdominal pain, and loose stools. A radiograph of the chest and platelet count were normal. The stools contained many acid-fast bacilli. The patient died 110 days after his original symptoms. The bacillus isolated from the stools was cultured by Dr. A. Q. Wells and found to be a normal human type tubercle bacillus of high virulence. Post-mortem examination showed an emaciated man weighing 45 kg. Small miliary tubercles were visible in many organs. There was a small area of consolidation in the mid-zone of the left lung about 4 cm. in diameter. The intestines showed gross tuberculous ulceration, while the portal glands were frankly caseous. The spleen (592 g.) was firm and had a finely granular appearance.

The histological appearances are those of miliary tuberculosis, but are remarkable for three reasons. There is necrosis rather than caseation. There are very many tubercle bacilli within the epithelioid and giant cells and lying free in the tissues. Fibrosis is minimal and the cellular reaction consists chiefly of polymorphs, most of which are degenerate. Epithelioid systems are not uncommon, but the cellular reaction generally is poor. The lungs show tubercles of varying ages as well as areas with necrosis with no tissue response. The spleen is packed with miliary non-reactive tubercles. There are tubercles in many other organs.

This man succumbed to miliary tuberculosis and tuberculous enteritis with a histological picture very like that of the non-reactive type nearly four months after an acute episode of thrombocytopenic purpura. The portal of entry was presumably the lung. It should be emphasized that the white cells were

numerically and morphologically normal throughout his illness.

Case 2.—F. I. N., a woman aged 59 years, began to lose weight and felt vaguely unwell 11 months before her death. These symptoms were slowly progressive. Pallor, fever, and an enlarged spleen were noted three months before her death. The haemoglobin was then 58% (8.6 g.%). The white cells varied between 1,000 and 2,500 per c.mm. while the differential count was normal and remained so throughout her illness. The platelet count was 70,000 per c.mm. Two sternal punctures showed marked hypocellularity with normal red and white cell maturation. The Westergren sedimentation rate was 60 to 120 mm. in one hour. She was given 3 pints of blood. The fever and wasting increased. The spleen enlarged to the brim of the pelvis. She died undiagnosed 11 months after the onset of symptoms, having pursued an apparently smooth course downhill with increasingly rapid deterioration towards the end.

Permission was given for a partial necropsy only. The spleen had a normal cut surface. The liver and mesenteric glands appeared to be normal. Histology of the liver and spleen showed vast numbers of tubercle bacilli matted into clumps, but the surrounding architecture was not disorganized (Figs. 1 and 2). Clumps of up to 15 bacilli could also be found inside normal liver cells and others were lying free in the sinuses. The absence of any inflammatory response or fibrosis was remarkable in both organs. Similar, but fewer, lesions were found in the marrow.

There is no definite evidence to indicate when the non-reactive tuberculous process began. Three months before death she had fever, splenomegaly, and granulopenia and the course of her illness throughout was a smooth one. Perhaps a chronic tuberculous infection in some organ not examined was responsible for most of her illness, but it seems more likely that the whole course was associated with a non-reactive type of lesion.

Case 3.—E. E., aged 25, became ill four months after his wife died of pulmonary tuberculosis, when he complained of pain in the chest, a productive cough, and night sweats. Seven weeks before his death a large pleural effusion was found. He became increasingly ill, breathless, and febrile. His sputum was repeatedly examined, but tubercle bacilli were seen only a few days before his death. A culture of the sputum, taken three weeks before death, grew *M. tuberculosis*. Three blood investigations may be selected as typical of many (see Table in next column).

Days Before Death	43	5	1
Haemoglobin (%) ..	82		57
Total white cells (per c.mm.)	32,000	82,000	15,000
Myeloblasts (%) ..	2·5	1	1
Promyelocytes (%) ..	2·5	2	1
Normal myelocytes (%)	30	26	1
Metamyelocytes (%) ..	—	14	11
Stab forms (%)	22	25	37
Polymorphs (%) ..	24	25	50
Lymphocytes (%) ..	12·5	5	8
Eosinophils (%) ..	—	1	—
Mononuclears (%) ..	6·5	1	1
Nucleated red cells per 100 white cells ..	—	—	4

The report on the sternal puncture by Dr. J. C. White emphasized the overwhelming white cell preponderance. Granulocytes at all stages of maturation were present, but some were morphologically abnormal. The few red cell precursors were normal. He considered this appearance compatible with subacute myeloid leukosis or leukaemoid reaction.

At necropsy the liver, spleen, and lungs showed numerous miliary tubercles. The lungs also showed nodules of caseous bronchopneumonia from 1 to 4 cm. in diameter. Some thoracic lymph nodes showed early caseation. The femoral marrow looked normal.

In the marrow there are a few scattered tubercles which consist of an area of necrosis containing a few epithelioid cells and nuclear remnants, but no giant cells. The tubercles in the liver are typically non-reactive with no surrounding tissue reaction whatever. No leukaemic deposits are present (Fig. 3). The spleen is riddled with tubercles consisting of necrotic areas with much nuclear debris, a few lymphocytes and epithelioid cells and a very few spindle cells, probably fibroblasts. The lung shows miliary lesions and massive confluent foci of acute necrosis. An occasional small collection of lymphocytes is the only cellular response. Sections, stained six years after death by the Ziehl–Neelsen method, do not show any tubercle bacilli.

Infection probably occurred six months before death and the lung was the probable seat of the primary infection or reinfection. The clinical picture was that of pulmonary tuberculosis and the leucocytosis was considered clinically in life to be a leukaemoid response to infection. Inspection of Figs. 3 and 4 shows that the tissue reaction approaches to a slight degree that found normally. As the causative organism was seen and cultured during life, it is curious that it cannot now be demonstrated in the sections.

Case 4.—M. R., a woman aged 54, gave a history of three years' illness with pulmonary tuberculosis, haemoptysis, and a pleural effusion which was repeatedly aspirated. Two months before death she suddenly experienced severe upper abdominal pain, subsequently found to be due to a large subcapsular haemorrhage in an infarcted area of the spleen. Examination revealed a large mass extending from the left ribs to the umbilicus and a just palpable liver. Repeated blood examination showed 6.7 m. red cells per c.mm.; haemoglobin, 14.0 g.%; packed cell volume, 57%; platelets, 350,000 per c.mm.; total white count, 30,000 per c.mm. (neutrophil polymorphonuclear leucocytes 85%, lymphocytes 12%, monocytes 3%). A very occasional myelocyte and nucleated red cell was also present. A sternal puncture produced an almost acellular smear with a differential count similar to that of the blood, but with the addition of about 10% of polychromatic normoblasts.

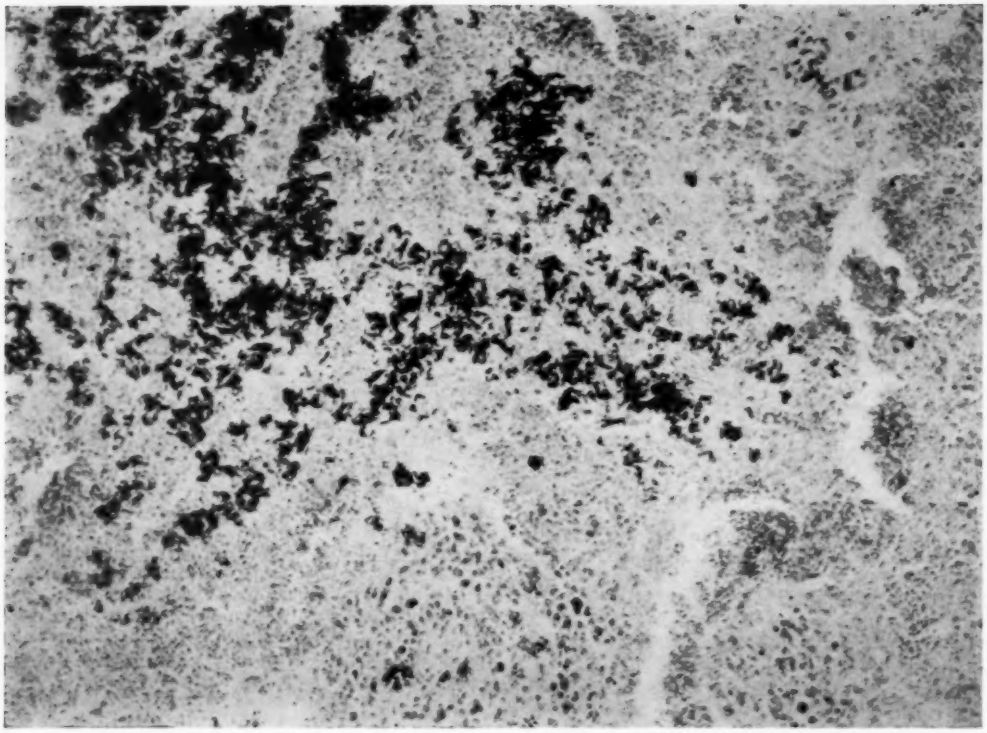

FIG. 1.—Spleen (× 200) stained by the Ziehl–Neelsen method, showing masses of tubercle bacilli and largely intact stroma with no tissue response.

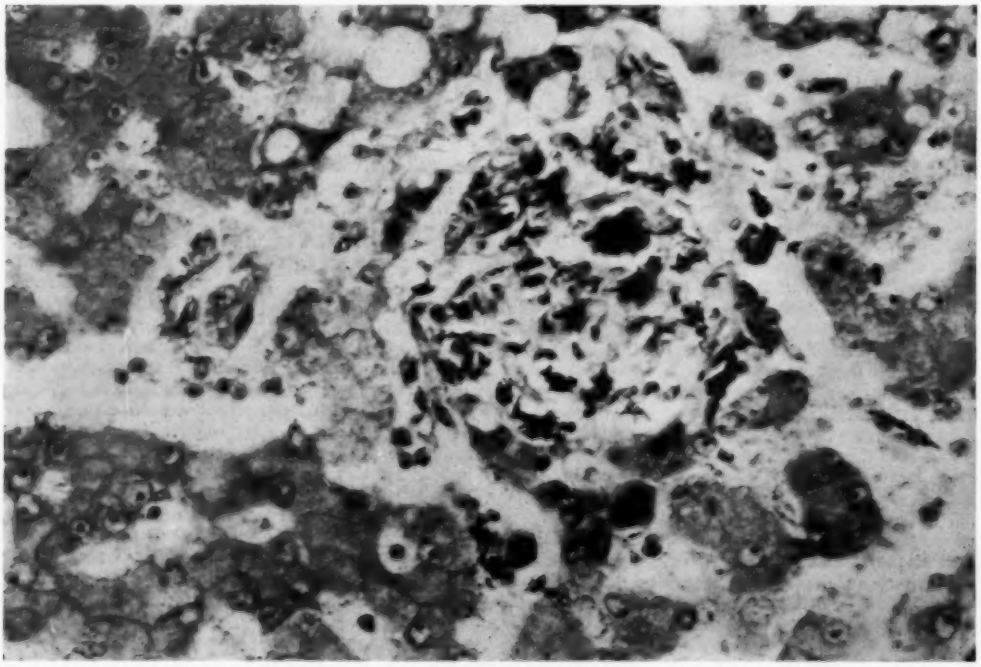

FIG. 2.—Liver (× 400) stained by the Ziehl–Neelsen method, showing small area of necrosis with tubercle bacilli; bacilli free in sinuses and in intact parenchymal cells.

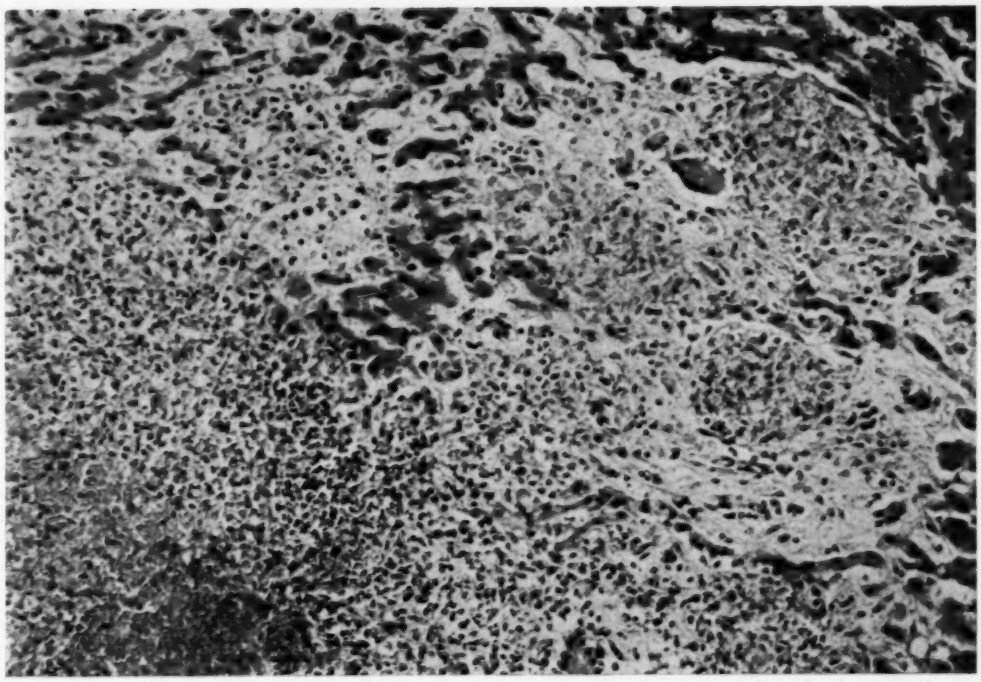

FIG. 3.—Liver (× 200) stained by haematoxylin and eosin. The tissue response consists of an occasional giant cell and some lymphocytes: large numbers of nuclear fragments also present.

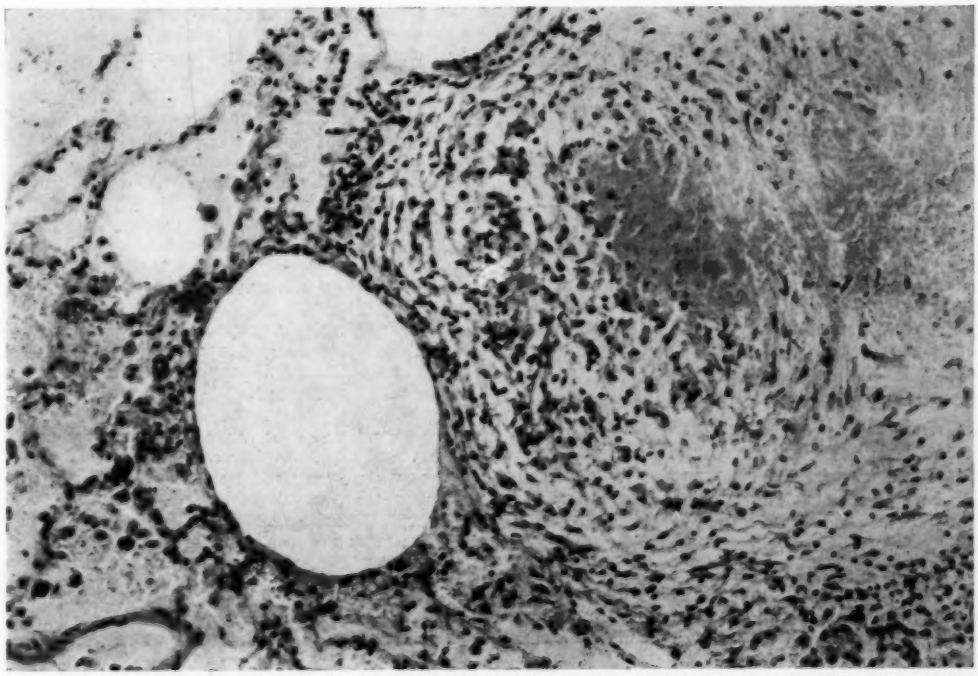

FIG. 4.—Lung (× 200) stained with haematoxylin and eosin, showing necrotic area with only minimal lymphocytic cuffing and some nuclear debris only.

This examination could not be repeated. No treatment was given and the patient improved considerably. Three days before her death she had repeated severe haemoptyses. She was then febrile, cachectic, and dying. A fixed fluctuant swelling had appeared over the left upper abdominal mass. A single blood examination showed haemoglobin 5.6 g.%, reticulocytes 6 per 100 red cells, platelets 380,000 per c.mm., total white cells 100,000 per c.mm. (neutrophil polymorphonuclear leucocytes 89%, lymphocytes 5%, polychromatic normoblasts 6%, no myelocytes seen). At necropsy the lungs were seen to be riddled with extensive bronchopneumonic spread of tuberculosis through all lobes with marked old cavitation and fibrosis. The small intestine contained two shallow ulcers extending horizontally round the gut, about three feet from its lower end. The spleen measured 30 × 20 × 18 cm. with a large fluctuant subcapsular haematoma overlying an irregular wedge-shaped infarct.

No tubercles are seen in the liver or spleen. The spleen, and to a lesser extent the liver, is heavily infiltrated with polymorphs, but no necrotic foci are present. A mesenteric gland shows typical normal tuberculous caseation. The marrow from several places shows increased cellularity with a preponderance of granulocytes. There is neither sclerosis nor fibrosis nor is the picture that of leukaemia. In none of the organs described so far were acid-fast bacilli demonstrated.

The lungs everywhere showed gross disorganization with much caseation and fibrosis and a scanty cellular response of the normal type. In contrast, but often adjacent, were areas of necrosis full of nuclear debris with no surrounding cellular reaction, which contained very many acid-fast bacilli. Adjacent to one of these areas was a small blood vessel containing red cells and polymorphonuclear leucocytes. There were a number of acid-fast bacilli free in the lumen of the vessel and inside polymorphonuclear leucocytes.

Sections through the ulcers in the small intestine showed in some areas the normal tissue response to the infection and contained no acid-fast bacilli. Adjacent to these areas were a few quite different lesions with intense polymorphonuclear leucocytic infiltration, nuclear debris, and a few lymphocytes, giving the appearance of tiny abscesses. There were very many acid-fast bacilli in almost all these areas. Adjacent were a lymph vessel and a blood vessel, the latter partly occluded by a cellular thrombus, both of which contained considerable numbers of intravascular acid-fast bacilli.

This patient had chronic pulmonary tuberculosis, and for at least two months she also had gross splenomegaly and a high red and white cell count. These findings cannot be explained by any ordinary haematological diagnosis and it is remarkable that the picture should have been associated with histological evidence at death of non-reactive tuberculosis in some organs. After years of a normal response to a tuberculous infection which was slowly advancing, the body's defences were suddenly overwhelmed, but in an unusual fashion.

The following four cases, very kindly given to me by Dr. F. B. Smith, were presented by him to the Association of Clinical Pathologists in 1949, but have not been previously published.

Smith: Case 1.—A man, aged 59, was admitted with generalized purpura and clinically was suspected of having typhoid fever. Clinical details are now lacking. His illness lasted for about one month. The total white cells numbered 3,000 per c.mm., but there was no record of the differential count.

At necropsy there was a thick exudate on the fauces and pharyngeal wall suggesting agranulocytosis. The pleural cavities were obliterated by old fibrous adhesions. The lungs appeared normal. The liver was slightly enlarged and contained sparse, ill-defined, small, pale yellow foci. The spleen was not enlarged and contained a few similar foci. The mesenteric glands were soft and matted together and contained fine miliary foci suggesting an acute tuberculous process.

The small foci in the liver, spleen, and portal glands showed necrosis with a complete absence of surrounding tissue response. They were packed with large numbers of acid-fast bacilli resembling *M. tuberculosis*.

This case appears to be a good example of acute typho-tuberculosis as it is described in the German literature.

Smith: Case 2.—A man, aged 52, became ill one month before his death. One week before death he was in a drowsy typhoid state with an intermittent rash, bleeding gums, and purpura. Repeated blood examination showed a haemoglobin of 75%, a total white count from 1,000 to 3,200 per c.mm. with only 500 to 800 polymorphs per c.mm. His condition steadily deteriorated until his death.

At necropsy an old scar from an empyema drainage wound was noted. The pharynx and fauces were extensively ulcerated. There were old fibrous adhesions in the right pleural cavity. The lungs contained small haemorrhagic foci. The liver (1,700 g.) and spleen (420 g.) contained small and inconspicuous pale yellow foci. A few small ulcers were present in the duodenal mucosa. A single tracheal gland was moderately enlarged. The marrow in the femur was essentially normal.

The liver, spleen, gland, and marrow showed miliary areas of necrosis with no tissue reaction containing acid-fast bacilli, occasionally in very large numbers. In the marrow bacilli were seen both in the necrotic areas and lying free among the normal marrow cells. There were some miliary necrotic foci in the lungs, which contained no acid-fast bacilli. Sections of these areas and of the duodenum were examined by Dr. J. T. Duncan, who confirmed the presence of mycelia and spores, and considered, in the absence of culture confirmation, that both sites were probably infected with monilia and that the lungs were infected with aspergillus as well.

Smith: Case 3.—A spinster, aged 65, four months before death had a macrocytic anaemia with a haemoglobin of 56%, and histamine-fast achlorhydria. The

total white cell count was constantly about 1,000 per c.mm. until her death and there were usually only 100 polymorphs per c.mm. while almost all the other cells were lymphocytes. There was no response to intensive liver therapy. A sternal puncture showed hypoplasia of the marrow with a gross preponderance of immature granulocytes. She was transfused, but relapsed. She was transfused again and the haemoglobin remained at 80% until death. One month before death she became febrile for the first time and irrational. She died after a slowly progressive illness lasting 10 months.

Necropsy showed a liver of normal size and a slightly enlarged spleen with, in both organs, scanty, inconspicuous miliary foci similar to those seen in the last two cases. Sections of the liver, spleen, and tracheal glands showed miliary, and occasionally confluent, areas of necrosis. These areas contained nuclear remnants, but with no surrounding cellular reaction. Large numbers of acid-fast bacilli were seen in all three sites.

This patient, with a 10 months' history, again raises the question of when she was infected with the tubercle bacillus. Was it a month before her death when she became febrile, or had she been infected from the beginning?

Smith: Case 4.—W. M. J., a man aged 45, had pleurisy as a young man. Five months before his death he apparently had pneumonia, with a haemoptysis and later a small hydrothorax. He had intermittent fever, recurrent, severe, staphylococcal abscesses, and went slowly downhill. Blood examination showed a haemoglobin of 55 to 65%; the total white cell count varied from 2,000 to 4,000 per c.mm. with 14 to 28% eosinophils. Sternal puncture showed hypoplasia of the granulocytic series. *M. tuberculosis* was not isolated in life. Post-mortem material was sent to Dr. A. Q. Wells, who grew and identified normal *M. tuberculosis* of human type. *Monilia* was repeatedly found in the sputum and mycelia were also present. These were identified culturally from post-mortem material by Dr. J. T. Duncan as *Aspergillus fumigatus*.

At necropsy the lungs showed a few small firm yellow nodules which contained the mycelia of aspergillus, but no tubercle bacilli. The spleen (430 g.) was dotted with white foci up to 3 mm. in diameter. The tracheal and para-aortic glands were enlarged and firm. Sections of the spleen and glands showed the characteristic areas of non-reactive tuberculosis. Large numbers of tubercle bacilli were present in these sites.

Entry was presumably by the lungs. The clinical course was conspicuous for the frequency and severity of the pyogenic infections. Both this and the mycelial infection were presumably due to the lowered resistance of the patient associated with the leucopenia. The evidence is not sufficient to prove whether the tuberculosis or the agranulocytosis came first. The fact that his first complaint was pneumonia and haemoptysis suggests that pulmonary tuberculosis was the first lesion.

Discussion

Are there sufficient common trends in the cases reviewed and reported to justify the concept of a single disease process? Undoubtedly the tubercle bacillus is common to all, but the variation in the reaction of the host is considerable. No cases have been included that had a normal tissue response to tubercle, but many cases have some features of the normal reaction, at any rate in some of the lesions. It seems probable that there are cases in which the tissue reaction is intermediate between the normal and the non-reactive types and that the cases under discussion merely represent an extreme degree of one type of tissue response.

The Parasite.—From the time when this condition was first recognized until recently there have been suggestions (Yersin, 1888; Loewenstein, 1934) that the causative organism was an avian type of tubercle bacillus. In no case has there been conclusive experimental proof of this assertion, and there are now many reports in which the causal organisms were proved to be a tubercle bacillus of the normal human or bovine types.

The Host.—There is considerable evidence that the tubercle bacillus is not immediately harmful to the normal living mammalian cell. For example, in tissue culture experiments Brieger (1949) grew explants of uninfected animals and found that when these explants were infected with tubercle bacilli some of the bacilli were "phagocytosed and contained in the macrophages for long periods with all the signs of a perfect symbiosis." It also seems that no extract from the tubercle bacillus is lethal or even toxic when injected into the living uninfected animal as a single dose. It must be assumed, therefore, that the tubercle bacillus does not normally kill by the manufacture of a cellular poison. When bacilli grow in the body they liberate products of metabolism that are treated as foreign proteins by the body. It is the nature of the sensitivity response of the body as a whole that largely determines the nature of the local tissue response to the local liberation of an essentially bland foreign protein. The fate of the patient now depends chiefly on the balance struck between the local tissue defence and the response of the body as a whole to the products of the bacillus, i.e., its sensitivity.

Bloch (1950) infected animals with three-day-old cultures of bacilli containing a high cord-factor content. Fifty per cent. of these animals died in 14 days compared with a 50% survival rate for 32 days in identical animals injected with three-week-old cultures. These results may be explained in a

manner differing from that put forward by Bloch himself. The three-day-old cultures are presumably growing logarithmically and producing from the moment they are injected large quantities of metabolic products. Normally the infecting dose is comparatively small, growth of the bacilli certainly is initially slow, and therefore the intensity and duration of the antigenic stimulus is different. It may be the time factors and the quantity but not the quality of the tuberculous products that produced the abnormal response in Bloch's mice.

In a normal person infected with tubercle there are few bacilli in the tissues and a vigorous local tissue response. One must assume that the sensitivity induced is the right kind to produce the vigorous local response. In non-reactive tuberculosis there are very large numbers of bacilli and no tissue response and therefore it seems very probable that the nature of the induced sensitivity is different.

The suggestion is therefore put forward that in human non-reactive tuberculosis it is the rate of liberation, or the quantity of the products of metabolism of the tubercle bacillus, that evoke a state of abnormal sensitivity in the patient. Alternatively or additionally, it is suggested that these people are in an abnormal state and cannot produce a normal sensitivity response.

An almost identical histological lesion occurs in voles (Wells and Robb Smith, 1946) infected naturally with the murine type of *M. tuberculosis* in an illness which, incidentally, may last several months. Similar appearances are also found in rats and mice infected intravenously.

The histological appearances of the lesions in transplacentally infected human babies are often similar. Two features of this infection seem to differentiate it from adult primary infection. First, the infection is blood-borne from the start and the dose is probably heavy, and secondly, and probably more significantly, the mechanism for the production of antibodies is imperfect in the neonatal period.

In some patients treated with cortisone for some quite different disease an unsuspected tuberculous lesion has advanced rapidly. The histology of these lesions is strikingly like that found in naturally occurring non-reactive tuberculosis. It appears that the probable explanation in these patients is that the mesenchyme cannot proliferate and differentiate in response to an appropriate stimulus. The histological appearance may in any case be attributed to paralysis of the primitive mesenchymal cell, and this explanation is supported by the observations that other stimuli of widely differing character cannot stimulate a response of the mesenchyme in animals treated with cortisone (Sissons and Hadfield, 1951).

The association of non-reactive tuberculosis with true leukaemia, which is usually chronic, is so frequent relative to the total number of reported cases of non-reactive tuberculosis that it cannot be explained by chance. In these cases it must be assumed that the leukaemia develops first and predisposes to this type of tissue response if the subject becomes infected with tubercle. Siegmund (1939) maintains that this type of lesion can occur only in a primary infection or in a second primary infection when the patient has antigenically completely recovered. Reference to Tables II, III, and the case reports show that in at least eight cases in the non-leukaemic groups and in all five of the leukaemic cases there was good evidence of an old active tuberculous infection.

Duration.—The histological appearance of non-reactive tuberculosis suggests an acute process, but many of the patients dying with non-reactive tuberculosis have been ill for months. It has already been mentioned that voles may survive with this histological picture for some months before they succumb. Clearly in most cases the non-reactive nature of the tuberculous lesion will only be established at death and so its duration must be surmised. The indirect evidence suggests that it may exist in man for some months.

Abnormalities of the Blood.—All but five of the reported cases of non-reactive tuberculosis in which a count was carried out had some disproportion of haematopoiesis. Arends (1950) has claimed that the common factor in non-reactive tuberculosis is an abnormality of the blood, and that this predisposed to an abnormal tissue response. The occurrence of this type of lesion in leukaemia, and possibly in myelosclerosis and Hodgkin's disease, in which the polymorphs are known to be abnormal, is then easily explained. There are, however, cases of non-reactive tuberculosis in which the polymorphs and all the other types of cell have remained normal throughout the course of the illness. Blood changes vary considerably and are most unlikely to be the cause of the abnormal tissue reaction, although this possibility cannot be excluded. There are, therefore, two possibilities. They may be brought about by the mechanical presence of the bacilli and the areas of necrosis in the marrow, but examination of the marrow shows that a very high proportion of the total volume of marrow is still histologically normal, so this explanation seems unlikely. The second possibility is a direct action of the products of metabolism of the tubercle bacillus on the marrow. It is now suggested

that the marrow cells are in a state of abnormal sensitivity, in a manner somewhat similar to the abnormal state already postulated for the cells of the other organs, and that it is this abnormal sensitivity of the body that explains the coincidence of non-reactive tissue response and the abnormal blood picture.

Aronson (1931) showed that tuberculin has a specific cytotoxic action on tissue cultures of the marrow and other tissues from infected animals and that the same small dose of tuberculin was innocuous to tissues from healthy animals. This work was largely confirmed by Moen and Swift (1936). Sensitivity of this type might explain the cases of agranulocytosis. Feldman and Stasney (1937) established experimentally that under certain conditions the products of metabolism of tubercle bacilli stimulated granulocytosis and produced a leukaemoid blood picture. The difference between stimulation and inhibition, particularly in this field of sensitivity, is probably slight, and the paradox of agranulocytosis and leukaemoid changes occurring in the same disease process may not be so improbable as first appeared.

This suggestive ambivalence of the reaction of the marrow to the products of tubercle bacilli finds some support in the work of Smith (1951) on the meninges. When she injected tuberculin intrathecally in cases of tuberculous meningitis, usually a violent cellular reaction with an outpouring of polymorphonuclear leucocytes was produced. In those patients who were Mantoux-negative no response was obtained.

Summary

Non-reactive tuberculosis is defined as a histological condition in which there are miliary areas of necrosis containing large numbers of tubercle bacilli surrounded by normal parenchymal cells or a minimal degree of the usual tissue response. There are 66 acceptable reports of this condition in the literature which is reviewed. The clinical course of the disease is most varied. It may be that of a rapidly overwhelming, typhoid-like illness or it may apparently continue for many months.

Its association with an abnormal blood picture is striking. Agranulocytosis, obscure anaemias, leukaemoid changes, and thrombocytopenia are frequent, and the condition is also found in patients with chronic leukaemia.

Eight previously unpublished cases are reported.

The mechanism of the abnormal tissue response and its association with an abnormal marrow response is discussed in the light of recent experimental work.

For permission to publish these cases I have to thank those doctors under whom they were admitted, namely, Professor L. J. Witts for Case 1, Dr. G. R. Steed and Dr. S. Noy Scott for Case 2, Dr. Nellan and Dr. J. C. White for Case 3, and Dr. W. A. Lister and Mr. G. L. Preston for Case 4.

I am also most grateful to Dr. F. B. Smith for permission to publish for the first time the four cases he presented to the Association of Clinical Pathologists in 1949; one of these, Smith Case 4, was actually investigated by Dr. H. G. Close, to whom I am also indebted. I also have to thank Professor T. F. Hewer's Department of Pathology for the photomicrographs.

I also am glad to acknowledge the kindness and help of Dr. E. M. Brieger, Professor G. Hadfield, Professor T. F. Hewer, Dr. R. G. MacFarlane, Professor Dorothy Russell, and Dr. A. Q. Wells.

Addendum

Since this paper was prepared, Friend and Thackerary (1952) have reported four cases of the association of tuberculosis and hepatosplenomegaly with an abnormal blood count. One case had typical non-reactive lesions. It has not been included in the tables.

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BACTERIOLOGICAL DIAGNOSIS OF TUBERCULOUS ENDOMETRITIS

BY

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Ever since chemotherapy for tuberculosis became available endometrial tuberculosis has aroused increasing interest amongst gynaecologists. The diagnosis of this condition, and also the assessment of progress during therapy, depends largely on the pathological examination of curettings or biopsy specimens. The isolation and identification of the infecting organism provides definite confirmation of the diagnosis. Histology may provide strong presumptive evidence, but it cannot be as definite as positive bacteriological findings.

Haines (1952) has reviewed the literature on the subject and has pointed out some of the difficulties of pathological diagnosis. The continual renewal of the endometrium allows little time for extensive lesions to develop, and the amount of tuberculous tissue in a specimen obtained by biopsy or curettage may be limited and probably unevenly distributed in the material available for examination.

This report deals with the examination of 600 specimens taken by biopsy or curettage between August, 1950, and June, 1953. Of the 600 specimens, 436 were from patients in whom the diagnosis was not confirmed by the laboratory. One hundred and sixty-four were taken at various times from 42 patients in whom a diagnosis of tuberculous endometritis was established. Sixty-nine of these 164 specimens were found to be positive by one or more of the methods employed, and 95 were negative by all methods.

The number of specimens examined from the cases of endometrial tuberculosis varied from one to nine. Of the 42 patients, 27 each produced one positive specimen, and in 10 of these cases it was the only specimen examined. Two positive specimens were received from each of eight patients; three positive specimens from each of four patients; four positive specimens from one patient; and five positive specimens from each of two patients. The intervals between specimens were usually about three months.

Technique

The specimens were sent to the laboratory in normal saline. Each specimen was divided into two approximately equal parts, one part being used for histological examination, and the other for culture and guinea-pig inoculation.

Histology

The histological examinations were made by Dr. A. H. T. Robb-Smith and Dr. R. H. Cowdell in the Department of Morbid Anatomy. Serial sections of the whole histological specimen were cut, and every fifth section was stained and examined. The histological diagnosis was based on the characteristic lesion and tissue reaction. No attempt was made to search for acid-fast organisms in the lesions.

Culture and Guinea-pig Inoculation

The other half of the specimen was ground in a Griffith tube with a volume of saline approximately equal to the volume of the specimen. Half of the resulting suspension, without further treatment, was injected subcutaneously into the thigh of a guinea-pig. The animals were tuberculin tested in the sixth week after inoculation, and were killed at the end of six weeks and examined for tuberculous lesions. In cases where the direct culture was negative, the organism was isolated from the guinea-pig lesions for sensitivity tests.

The remainder of the tissue suspension was mixed with an equal volume of 4% NaOH in a universal container. It was kept at 37° C. in the incubator for 30 minutes, and shaken at five-minute intervals. It was then diluted with five volumes of sterile distilled water and centrifuged at 3,000 r.p.m. for 30 minutes. The supernatant fluid was discarded. One drop of phenol red indicator solution was added to the sediment, and the mixture was brought to *p*H 7.4 with dilute HCl. Culture media were then inoculated with the deposit. The media in routine use were Löwenstein-Jensen and a modified Kirschner (Vollum, 1952). Dorset egg was also used in case any bovine strain should fail to grow on the glycerinated media. No strains were found which grew only on Dorset egg. All cultures were incubated at 37° C. for six weeks, and were examined at weekly intervals. All cultures, whether obtained directly

TABLE I

ANALYSIS OF THE RESULTS OF EXAMINATION OF 164 SPECIMENS OF ENDOMETRIUM OBTAINED BY BIOPSY OR CURETTAGE FROM 42 PATIENTS WITH PROVED TUBERCULOUS ENDOMETRITIS

Method of Examination	Combinations of Negative and Positive Results														Total of All Positive Specimens
Histology	+	+	0	+	+	0	0	+	0	+	0	0	0	0	35
Guinea-pig inoculation	+	+	+	0	+	+	0	0	+	0	+	0	0	0	40
Kirschner culture	+	+	+	+	0	+	+	+	0	0	0	+	0	0	33
Löwenstein-Jensen culture	+	0	+	+	0	0	+	0	+	0	0	0	+	0	24
No. of specimens	7	2	8	1	6	3	5	4	2	15	12	3	1	95	69

or via the guinea-pig, were eugonic, and cultural characteristics were of the human type.

Results

Table I shows the various combinations of positive and negative results found in the 69 specimens which were positive by any of the four methods employed—histology, guinea-pig inoculation, Löwenstein-Jensen culture, and Kirschner culture. Only seven specimens were positive by all four methods. Guinea-pig inoculation gave slightly better results than other methods, with 40 out of 69 positive. Histology revealed 35, Kirschner culture 33, and Löwenstein-Jensen culture 24. Löwenstein-Jensen medium failed in 12 specimens where the Kirschner medium was successful, and was positive in three specimens where the Kirschner medium was negative. The two culture media together revealed 36 of the 69 positives. Ninety-five specimens were negative by all methods. The diagnosis was made on histology alone in 15 cases, on guinea-pig inoculation alone in 12 cases, and on culture alone in four cases.

The discrepancy between the four methods may be due to a number of factors, but probably chiefly to the small amount of tuberculous tissue and the small number and uneven distribution of viable tubercle bacilli in any specimen. If the whole of each specimen had been used for any one method, a higher percentage of positive results by that method might have been expected, but each method has certain disadvantages, and the risk of missing a positive finding is probably less by dividing the specimens and employing different methods.

Histology has the advantage of providing the result more quickly, but positive findings by guinea-pig inoculation or culture are more reliable.

Summary

Six hundred specimens of endometrial curettings or biopsy were examined for tuberculous infection by histology, guinea-pig inoculation, and culture. Sixty-nine of these specimens were found to be positive by one or more of these methods. Histology detected 51%, guinea-pig inoculation 58%, and culture 52%.

No single method should be relied on for the diagnosis of tuberculous endometritis.

Mr. John Stallworthy kindly provided the majority of the specimens for examination.

I am indebted to Dr. A. H. T. Robb-Smith and Dr. R. H. Cowdell for the histological reports on these specimens.

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OXYURIS GRANULOMA OF THE ENDOMETRIUM

BY

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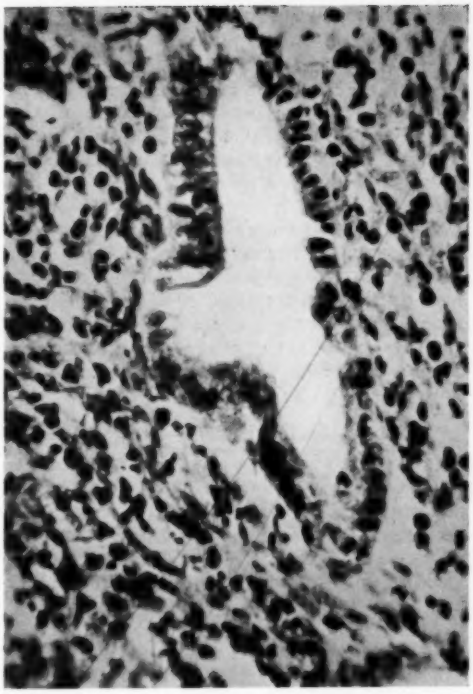
The first description of an intrinsic genital lesion caused by threadworms (*Oxyuris* or *Enterobius vermicularis*) is by Klee (1920), who found a gravid female worm in a granulomatous lesion of the cervix. There are a few other reports, reviewed comprehensively by Symmers (1950), of the identification of threadworms in other parts of the female genital tract, and search of the literature since Symmers's review has brought to light four further studies of oxyuris infestation of the female genitalia (Fatheree, Carrera, and Beaver, 1951; Freudenberg, 1951; Leschke, 1951; Gill and Smith, 1952). It is interesting that the majority of such reports are in the German literature, which may be a reflexion of the very high incidence of oxyuriasis found in Germany. Between 71 and 97% of German schoolchildren are affected (Mendheim and Scheid, 1948; Beckers, 1949, and Ebert, 1949, quoted by Neumann and Wiedemann, 1950) as compared with 40 to 55% in similar groups in this country (Young, 1942; Mac Keith and Watson, 1948). The findings in the published cases of genital oxyuriasis indicate that the worms (adult gravid females) find their way into the genital tract during their nocturnal wanderings over the perineum. They gain access to the uterus, Fallopian tubes, and peritoneal cavity by crawling up the lumen of the genital tract. In a case encountered recently such a worm had lodged in the endometrium, where it caused a granulomatous lesion which was discovered during the routine histological examination of uterine curettings.

Case Report

The patient was a married woman of 45 who had had a uterine prolapse since the birth of her last child nine years previously. The only symptoms she had which might have been related to the threadworm infestation were pruritus and menorrhagia of six months' duration. The latter could also be explained by the presence of multiple fibroids of the uterus. On examination, the external genitalia were found to be healthy but the vaginal mucosa was hyperaemic and the cervix and body of the uterus were swollen and tender; there was a small inflamed area at the cervical lip. A diagnostic curettage was performed a few days before the uterus was removed.

Curettings.—The endometrium was in the premenstrual phase and its stroma was heavily infiltrated by eosinophil granulocytes (Fig. 1). Fortuitously, the histological preparation contained a small granulomatous area, 4 mm. across, in the centre of which were some parasitic remains (Fig. 2). Serial sections were cut from the rest of the block; a few were stained with eosin and methylene blue or by Mallory's connective tissue stain, and the remainder with haematoxylin and eosin. It was found that the granuloma consisted of a central space containing parasitic eggs which had an asymmetrical outline and were approximately 50×20–$30\ \mu$ in size. They consisted of a hyperchromatic central mass surrounded by a thick transparent envelope; larval outlines could be recognized in a few. The egg-shells did not develop a blue colour with Mallory's stain, which indicated that they were not chitinous. These features are characteristic of oxyuris eggs. Dispersed among the eggs were a few fragments of tissue, apparently visceral remains, and round about the central collection was a hyaline eosinophilic capsule in which some nuclear structure could be seen. The capsule developed a deep blue colour with Mallory's stain, suggesting a chitinous composition; it was identified with certainty as the cuticle of the worm by the demonstration of an oesophageal bulb in direct continuity with it in a deeper section (Fig. 3), and in other sections by the recognition of a lateral crest. The presence of nuclei in the cuticle indicated that the worm at the time of the curettage was either alive or very recently dead. The portion of the worm at our disposal measured 3.5×0.4 mm., but the complete worm may well have been somewhat larger than this.

The granulomatous reaction around the worm consisted of a fairly well demarcated zone, 1 mm. thick, in which there were large numbers of degenerating eosinophil and neutrophil granulocytes together with lymphocytes and plasma cells. The granulation tissue close to the worm showed the greatest degenerative changes and contained a few strands of fibrinoid material. Around the granulomatous area the endometrial stroma was heavily infiltrated by inflammatory cells, among which eosinophils were again particularly abundant. The endometrial fragments not directly connected with the granuloma showed only an infiltration with eosinophil granulocytes. Encapsulation by fibrous tissue, calcification, foreign-body giant cells, and Charcot–Leyden crystals, which are said to be common in oxyuris granulomas, were not seen. Follicle formation in the



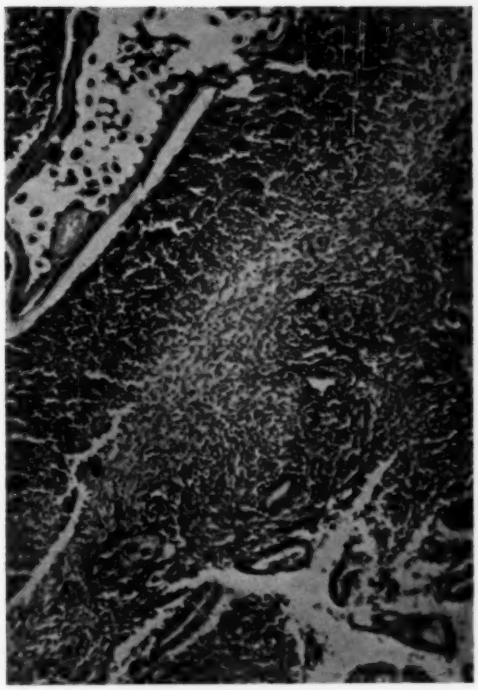


FIG. 1.—Secretory endometrial gland in a stroma which is heavily infiltrated by granulocytes, mostly eosinophil in type. (Haematoxylin and eosin. ×380).

FIG. 2.—Gravid female threadworm surrounded by necrotic granulation tissue, with adjacent endometrial glands and infiltrated stroma. (Haematoxylin and eosin. ×75.)

FIG. 3.—High-power view of worm showing capsular nuclear material, oesophageal bulb (top left), and typical oxyuris ova. The adjacent granulation tissue contains large numbers of eosinophil granulocytes. (Haematoxylin and eosin. ×380.)

granulation tissue has also been previously described and this feature was recognized in a few of the sections.

Uterus.—After the diagnostic curettage the patient had a menstrual period, following which the prolapse and fibroids were treated by total hysterectomy. The uterus was examined by us for any sign of threadworm infestation. It weighed 450 g. and contained a large intramural fibroid, 5 cm. across, and a few smaller fibroids of less than 1 cm. diameter. There was no macroscopic or microscopic sign of threadworm infestation in the endometrium or in the lumen. Histologically, the endometrium was in the early follicular phase; the stroma showed a moderate infiltration by leucocytes, mainly eosinophil granulocytes, and no other pathological feature. The fibroids were cellular but benign.

Stools and Perianal Swabs.—The stools were examined once and "cellophane" perianal swabs were examined on four occasions with negative results. Direct questioning of the patient about a history of threadworms, did, however, elicit the surprising information that she had suffered from worms on and off for over 30 years but had made no effort to obtain treatment since leaving school. She had last observed worms in her stools "during the summer," i.e., about two months before her admission to hospital, and there had been no sign of them since.

Discussion

This case illustrates the capacity of the threadworm to live an almost harmless parasitic existence for very many years. Even when the uterus was invaded there was apparently little threat to health. More important from the pathological aspect is that the case may allow certain conclusions to be drawn on the time required for an oxyuris granuloma to develop. It is likely that the worm crawled into the uterus just after the last menstrual period and that it was then enveloped by the proliferating endometrium. Fortunately the curettage was done just before the next period and the worm was not lost in the menses as it otherwise might have been. The time between the final day of the last period and the curettage was three weeks and it is suggested that the histological picture required no longer than this to develop. This short history was probably responsible for the failure to produce giant cells, calcification, and a fibrous capsule. The case also calls attention to a possible cause of infiltration of the endometrium by eosinophil granulocytes. Having regard to the minute size of the worm compared with the extent of the endometrium, it is a cause which might well be overlooked.

Summary

A granuloma containing a gravid female threadworm was found during the routine histological examination of uterine curettings. The endometrial stroma showed a striking infiltration by eosinophil granulocytes. It is believed that the granuloma reached its full development in not more than three weeks.

Our thanks are due to Dr. R. M. Bernard and Dr. A. T. Forbes for supplying the clinical details.

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MALIGNANT GIANT-CELL SYNOVIOMA OF PHALANX

BY

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Primary sarcoma of a phalanx is uncommon. Osteogenic sarcoma, fibrosarcoma, and chondrosarcoma have been infrequently reported, but a search of the literature has not revealed any previous record of a primary malignant synovial tumour arising endosteally, although benign synoviomata are fairly common in the digital soft tissues.

The unusual site of origin of the tumour described here is suggested both by the clinical and radiological findings. Invasion of bone by synovial tumours of digits and other sites has been reported by Wright (1952), Fletcher and Horn (1951), and by others, but in such examples the involvement generally appears to be either the result of pressure atrophy or due to direct extension of a parosteal growth. Certain primary expansive tumours of phalanges and metacarpals have been described (*vide infra*), which characteristically produce a rich osteoid matrix. These are usually classified with osteogenic fibroma of bone and present a histological picture which in some ways resembles the less cellular parts of the tumour reported here.

Case History

Mrs. N. F., aged 35, a housewife (BTR/511), in March, 1952, fell from a chair while hanging out washing. She fell on to the outstretched right hand, injuring the fifth finger. Before this there was no evidence of any bony or arthritic changes. Following the accident, the finger became painful and swollen but no medical advice was sought. As the symptoms were progressive, the patient consulted her doctor in July, 1952, who referred her for surgical advice. On examination at that time there was a deformity of the fifth right finger, with a tender swelling of the middle phalanx region.

X-ray examination on July 19, 1952, showed an extensive cystic swelling of the intermediate phalanx, with expansion and marked thinning of the cortex (Figs. 1, 2*a*). There may have been a former fracture of the phalangeal shaft in the antero-medial region. Apart from a very slight periosteal reaction, there was no sign of any new bone formation. There appeared to be perforation of the thinned cortex antero-externally. The other two phalanges were normal, as also were radiographs of the skull and other bones. The finger was amputated at the metacarpo-phalangeal joint in September, 1952.

FIG. 1.—Radiograph of right hand on July 19, 1952, showing the well-marked expansion and thinning of the cortex of the middle phalanx. By September the transverse bony septum had been reduced to a thin bundle of collagen fibres.

Amputation Specimen.—The finger when received had already been partly cut sagittally. It showed an irregular spheroidal mass of solid pale yellow-white tissue approximately 1.5 × 1 cm. occupying the middle segment of the finger in place of the intermediate phalanx (Fig. 2*b*). This solid tissue cut easily with the knife and was of a uniform rubbery consistency. It was fairly well circumscribed, more especially upon the volar aspect, and consisted of a main mass formed of a number of

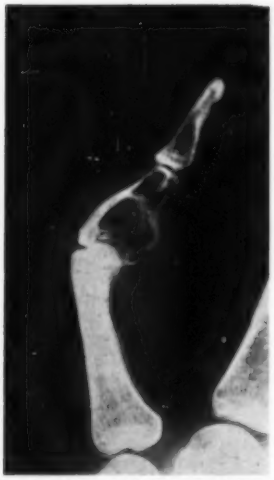

FIG. 2a.—More oblique view of the finger, July 19.

FIG. 2b.—Reconstruction of finger from three paraffin sections. × 1. The central part is in a plane slightly lateral to the proximal and distal sections.

FIG. 2c.—Radiograph of surgical specimen, the left portion being comparable with Fig 2b (lateral half of finger). Note further loss of normal bony shadow.

ill-defined, rounded nodules fused together (A, Fig. 2*b*). There was also an adjacent distal part (B, Fig. 2*b*) of rather deeper hue, attached to, but separated from, the main mass by a transverse band of dense collagen. The flexor profundus tendon could be identified, but appears to have been invaded deeply by the tumour at C (Fig. 2*b*). The distal phalanx was decalcified *in vivo* and offered but little resistance to the knife. No naked-eye abnormality was noted in the proximal phalanx.

A radiograph of the specimen (Fig. 2*c*) showed almost entire destruction of the intermediate phalanx, and that the proximal two-thirds of the distal phalanx was markedly radiolucent, although a dim "ghost" of this bone could still be discerned. The proximal phalanx was normal except for some loss in density indicating decalcification.

Histological Examination

The entire finger was sectioned sagittally in three blocks; but section No. 2 (middle) was at a somewhat lateral plane to Nos. 1 and 3 (Fig. 2*b*). Both paraffin and frozen sections were cut. These were stained by haematoxylin and eosin, haematoxylin, phloxin and tartrazine, Gömöri's silver impregnation, periodic-acid-Schiff, sudan III, sudan IV, alkaline methylene blue (*p*H 9.0), and Perls' method for iron. Both stained and unstained sections were examined by polarized light.

Section 1: Proximal.—There were early degenerative changes in the bone of the phalanx shaft. Both articular cartilages and their related synovia showed evidence of early degenerative arthritis. There was no neoplastic invasion of the phalanx, but dorsally a few tumour cells were tracking along the peritendinous tissue.

Section 2: Middle.—Both flexor and extensor tendons were distorted and invaded by tumour cells, the tendon sheaths showing early proliferative changes. The distal articular cartilage was fairly well preserved together with a thin sliver of subchondral bone and a short fragment of ventral shaft cortex. The major part of this section consisted of a mass of fibro-cellular tumour tissue which had an ill-defined nodularity, and a few small fragments of incompletely absorbed mature bone were found lying between the tumour cells. Ventrally, the tumour was bounded by a layer of dense collagen fibres, probably representative of the displaced periosteum. This limiting layer was less well defined dorsally where it had been invaded by the tumour. Distally, there was also a well-defined transverse band of collagen fibres which enclosed the main mass of the growth, and which itself was probably the residue of the bony transverse septum seen in Fig. 2*a*. The main tumour consisted of irregular spheroidal and fusiform cells lying in a rich stroma of dense collagen fibres (Figs. 3 and 4). There were also numerous bizarre giant cells mainly around the peripheral areas of the ill-defined nodules. Many of these giant cells were heavily laden with small globules of sudanophil material (Fig. 5). In unstained frozen sections, the globular material showed birefringence of a low order. No steroid crystals or spherulites were found. It was generally

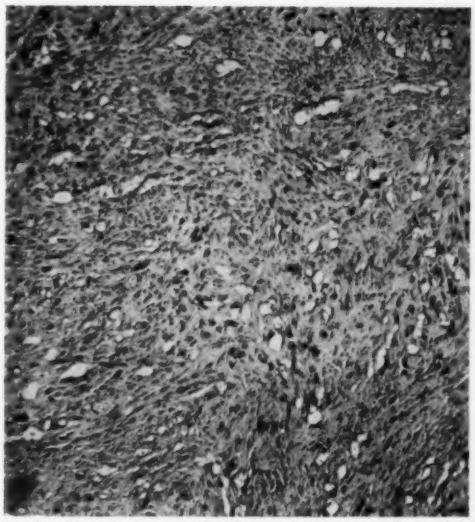

FIG. 3.—General structure of main tumour mass towards proximal end of residual shaft of intermediate phalanx. Haematoxylin and eosin, ×46.

FIG. 5.—Giant cells from edge of tumour nodule heavily laden with small sudanophil globules. Sudan iv, × 100.

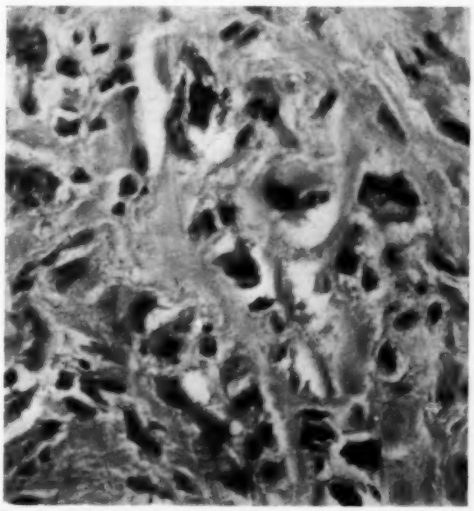

FIG. 4.—Cellular detail of main tumour mass. The cells are mainly small and spheroidal or polyhedral, together with a few bizarre giant cells. There is a well-developed collagen stroma. Haematoxylin and eosin, × 370.

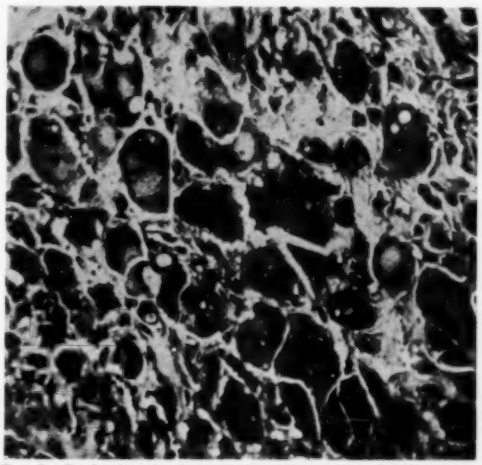

FIG. 6.—Predominantly giant celled area from edge of main tumour mass. The cytoplasm of the paler cells is stained mainly with tartrazine, and shows marked vacuolation. Haematoxylin phloxin, and tartrazine, × 210.

observed that the sudanophil material was minimal or absent from those giant cells showing mitosis or cytoplasmic vacuolation. The dense stromal collagen showed typical birefringence and its form mainly suggested new fibre formation by the tumour cells rather than residual collagen matrix from destroyed bone, cartilage, or tendon. In certain areas there was a suggestion of a trabecular arrangement.

Lying between the collagen fibres were numerous round or oval "clefts," in some instances forming larger spaces by confluence. It had originally been thought that many of these were fat cells, but in frozen sections these spaces were again empty and

devoid of sudanophil material. Neither in paraffin nor frozen sections stained with methylene blue and by the periodic-acid-Schiff method was any evidence found of a mucinous content in these clefts, and one can only conclude that they must have contained a water-miscible fluid which had been lost into the formol fixative.

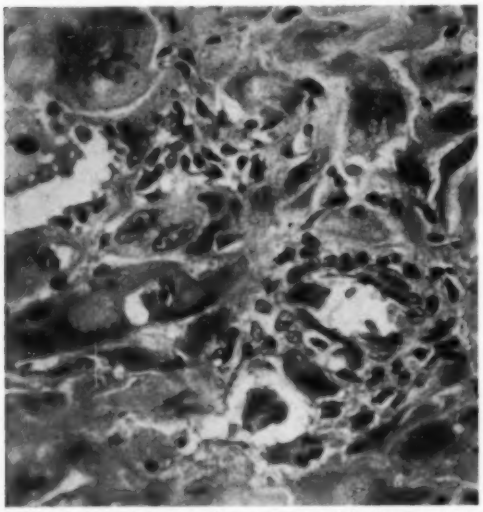

FIG. 7.—Tumour tissue from distal outlying nodule showing numerous giant cells, some simulating atypical osteoclasts. There are a few small spindle cells present, but only scanty stroma. Haematoxylin and eosin, × 370.

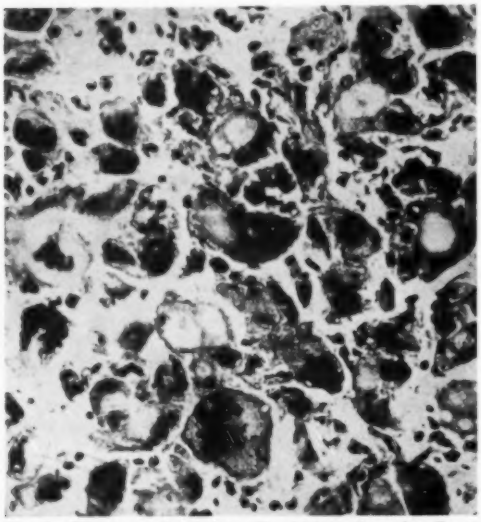

FIG. 8.—Bizarre vacuolated giant cells which are the main cell component of the distal outlying nodule. Haematoxylin, phloxin, and tartrazine, × 240.

It was possible to observe the manner in which these small clefts were formed. The earliest evidence was to be found in the foamy cytoplasm of the larger multinucleated giant cells. Small vacuoles appear to form which grow, and a number of clefts of moderate size were noted in which there was still a thin rim of granular cell cytoplasm, with the deeply stained pyknotic nuclei compressed and aggregated to one side (Figs. 4 and 6). Occasionally, such clefts or vacuolated giant cells were surrounded by a thin phloxinophil rim of material which showed feeble birefringence. This latter feature is probably due to protein crystallization at a gel/fluid interface, and is essentially similar to the early radial zone of osteoid which may be seen around the periphery of the mature cartilage cells of an orderly column of epiphyseal chondrocytes, and may represent the earliest sign of endochondral ossification. It was generally noted that the giant cells which showed this cytoplasmic vacuolation stained metachromatically a pale mahogany colour with the phloxin-tartrazine staining, whereas those giant cells in which there were no vacuoles were typically phloxinophil. (These tissue clefts are generally typical of synovioma, and may be numerous and large even in the absence of any giant cells. Obviously, the manner of cleft formation indicated here may not be the only one; on the contrary, the modified cell structure seen in the central fibrous areas of this tumour suggests that maturation of the giant cells, with concomitant morphological changes, is associated with a well-marked collagen stroma and numerous clefts. Hence, in certain synoviomata these latter features may be the more prominent.)

There were also quite a number of larger spaces lined with flattened cells whose appearance suggested dilated lymphatics. There was little or no sudanophil material lying between the stromal collagen fibres, nor yet between the fibres of the limiting collagen bundles and the adjacent tendons. This latter feature is usually well marked in giant-cell tumours of tendon sheaths.

In the cells of the main tumour mass, mitoses were numerous and often abnormal. The mitotic ratio (Price, 1952) was 97 to 1, and in many high-power fields 3% or more of the cell nuclei were in mitosis. No necrosis was seen, and no evidence found of any new bone formation in the collagen matrix, even in the denser areas where it resembled osteoid. Many of the giant cells were multinucleated, and transitional forms were noted to be fairly typical osteoclasts (Figs. 6 and 7).

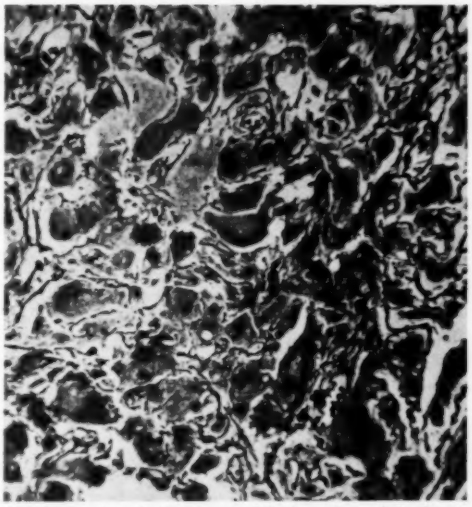

FIG. 9.—Scanty reticulin network of distal outlying nodule of tumour. This appearance was also characteristic of the peripheral parts of the main tumour mass which were predominantly giant celled. Gömöri silver method, × 190.

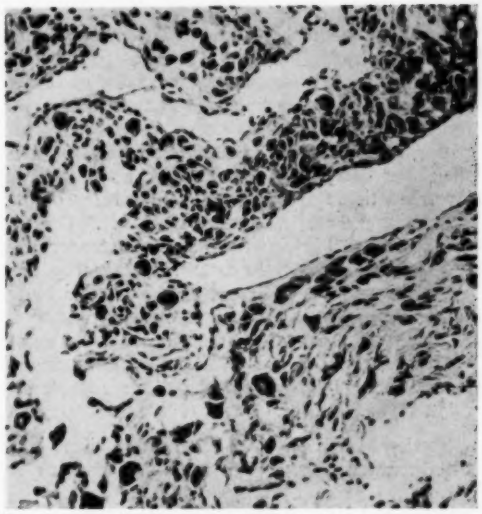

FIG. 11.—Area of tumour near residue base of intermediate phalanx. There are a number of large complex spaces lined by flattened cells suggesting a synovial structure. Haematoxylin and eosin, × 180.

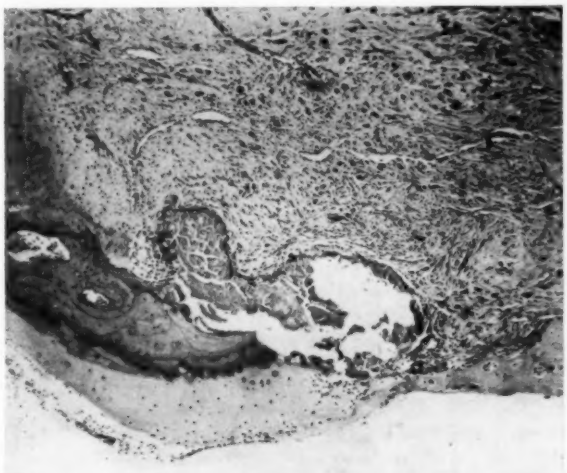

FIG. 10.—Area of degeneration of cartilage matrix at the edge of residual fragment of proximal articular cartilage of intermediate phalanx. Haematoxylin and eosin, × 46.

The distal part of the tumour (Fig. 2*b*) lay in the concavity formed by the residue of the head of the phalanx. It consisted almost entirely of atypical multi-nucleated giant cells similar to those of the peri-nodular areas described above (Fig. 8). These cells mainly showed marked cytoplasmic phloxinophilia, with five to 10 irregular pyknotic or vesicular nuclei. In many cells the deeply staining irregular nuclei were indistinct and conjoined, an appearance suggesting either incomplete separation, atypical nuclear lobation, or fusion of an aggregated mass of the smaller nuclei observed in other cells. Nucleoli were, in general, indistinct. Many of the cells resembled atypical osteoclasts and showed coarsely granular cytoplasm, a few having small vacuoles. Mingled with these cells were smaller numbers of spheroidal and fusiform cells mostly related to the small blood capillaries. The intercellular stroma was scanty, consisting of a few reticulin and collagen fibres, together with a small amount of amorphous collagen (Fig. 9). Occasional mitoses were seen but were far fewer than in the main tumour mass.

Three main residual fragments represented the proximal articular cartilage of the intermediate phalanx. These showed a thinned shell of old subchondral bone. The cartilage was degenerate, and there were areas of amorphous granular material apparently derived from the disintegrated juxta-osseous zone of the articular cartilage (Fig. 10). The granular debris was isotropic, strongly phloxinophil, did not stain metachromatically with methylene blue, but showed a strongly positive reaction with

the periodic-acid-Schiff method. Surrounding this material was reactive tissue, including a few cells of osteoclastic type. No neoplastic cartilage was found anywhere in the tumour. The synovium of the proximal interphalangeal joint showed early villonodular changes but no iron-containing or other pigment was found. In the vicinity of these cartilaginous fragments were a number of large branched tissue spaces lined with a layer of cells simulating normal synovium (Fig. 11). Deep to this surface cell layer were numerous tumour giant cells. It is difficult to be certain whether this represented invasion of displaced joint synovium by the tumour, or whether the structure was proper to the new growth. A close examination of the branched spaces suggested a relationship to the larger ones mentioned above, which had been regarded as dilated lymph spaces.

Section 3: Distal.—The bone and cartilage of the distal phalanx showed degenerative changes but were not invaded by tumour cells.

Comment

The tumour described here appears to have a double morphology: (1) areas, mainly giant celled, with very scanty stroma (Figs. 6 and 8); (2) other parts, usually central, showing fewer giant cells but with fairly numerous smaller spheroidal and fusiform cells associated with numerous small tissue clefts and much tumour collagen or osteoid (Figs. 3 and 4).

The cell pleomorphism, the degree of mitotic activity, and the invasiveness indicate malignancy, and although apparently of endosteal origin, the tumour should be regarded as a synovial sarcoma of giant-cell type.

The osteoid-forming expansive tumour of a metacarpal bone reported by Jaffe and Mayer (1932) may have had a similar origin and be closely related to the one reported here. The former, however, consisted mainly of spindle and spheroidal cells producing much trabecular osteoid ("coarse, irregular decussating, fibres which stained like collagen"). This osteoid was partly calcified, partly ossified. These authors mention the complete absence of tumour giant cells and relate their tumour to the "osteoid chondroma" of Virchow (1863) also reported by others, mainly in the German literature. By virtue of the ability of such a tumour to form osteoid and/or bone the malignant counterpart would naturally fall within the broad class of osteogenic sarcoma. Jaffe and Mayer express the opinion, moreover, that had surgical removal of this growth and the metacarpal bone been long delayed it would have undergone malignant changes and have developed into true osteogenic sarcoma. Such a view is conjecture based upon experience and judgment, but nevertheless it implies that the osteoid-producing tumour was to be regarded as a presarcomatous lesion. Lichtenstein (1952), who mentions this particular tumour in his chapter on "Osteogenic Fibroma of Bone," comments further upon the rarity of this type, and relates such neoplasms to the bones of the hand and foot in particular. He also points out their slow rate of growth and characteristic bone expansion.

Irrespective of its malignancy, the present tumour appears to be able both to produce and destroy osteoid, the change in function being accompanied by modified cell morphology as seen in the two forms noted above. It is of interest to note that the intracellular sudanophil material appears in the collagen-destroying giant cell areas, while, on the contrary, it is generally absent in the more central areas of dense collagen stroma, smaller cell size, cytoplasmic metachromasia, and tissue cleft formation. It seems probable that with further maturation of the stromal collagen of these central areas some degree of ossification would have taken place. The nodular appearance of the tumour (Fig. 2*b*), while not well defined, is very similar to that depicted in synoviomata by Wright (1951); in fact, this author's low-power photomicrograph (Fig. 376, Wright, 1951) is a very close image. In Wright's series of 57 benign giant-cell synoviomata of fingers and toes there is no mention, however, of invasion and expansion of the adjacent bones. His paper does illustrate the typical pressure atrophy of adjacent bone which may be caused by a benign giant cell synovioma especially when occurring in the foot, but is unusual in the finger growths. This latter point is also made by Coley (1949), and others. Nevertheless, malignant synoviomata, of which about one half occur around the knee, not infrequently erode and destroy adjacent bone but do not usually cause cortical expansion.

The recurrent synovioma of the finger described by Wright (1949) shows some histological features in common with the present tumour, but in its recurrent malignant form seems to have been a more cellular growth, predominantly of spheroidal cells with relatively scanty stroma. This latter form has been seen in two other cases in the Bristol Bone Tumour Register collection (BTR/7 and BTR/426). The latter recurred eight months after surgical excision.

The case reported by Berger (1938) is of great interest, since it was essentially a fibrosarcoma with

areas suggesting typical synovioma. The reverse of these conditions has also recently been seen in a tumour from the knee region by one of us in a man aged 31 (BTR/597). The tumours reported by De Santo, Tennant, and Rosahn (1941) are described as of mixed cellular structure, showing polyhedral and spindle cells with foci of numerous giant cells. Their patient (No. 11) died with terminal pulmonary metastases.

Fletcher and Horn, in describing the features of bone involvement by giant cell synovioma, mention destruction of bone by erosion with subsequent tumour growth within the marrow cavity. The radiographs illustrated by these authors certainly show well marked focal osteolysis with cortical perforation, but only very questionable evidence of cortical expansion (Case No. 1). In their reported cases there was an extra-osseous soft tissue tumour in three instances (Nos. 1, 7, and 8), the radiological appearances clearly suggesting invasion of bone from without. Erosion of adjacent bone ends is well shown in the radiograph of their Case 2 described in this series.

Vermooten (1925) has reported a benign central osteolytic phalangeal tumour under the term "xanthosarcoma." This was described as having been of a bright yellow cast, and contained numbers of "foamy" cells together with a few giant cells intermingled with the fibrous stroma. In addition to the central area of marked bone destruction there were a number of small cortical erosions, and a soft tissue tumour. These findings, in the light of the relevant case history, suggest bone invasion from without, the tumour being probably a giant cell synovioma with an unusually large "foamy" cell component.

Clark (1952) reported an osteogenic sarcoma of phalanx, which appears to have been primarily a fibrosarcoma with evidence of both osteolytic and osteoblastic activity. It appears to have invaded the distal and intermediate phalanges from without, and the history and published skiagrams suggest a parosteal origin.

Histogenesis.—King (1952) rightly draws attention to the insecure basis of the presumed origin of synovioma. Tumours showing the structure of synovioma but unrelated to anatomical synovium have also been reported by Fisher (1942), Briggs (1942), and by Haagensen and Stout (1944). The histogenesis of these atypical growths can be open to only two explanations: (1) origin from heterotopic synovium, or (2) origin from pluripotential mesenchymal cells.

Appearances suggest that the giant cells of the present tumour are associated with destruction of collagen, being related functionally and morphologically to osteoclasts. In a paper on the Strangeways Collection, Lawford Knaggs (1932) briefly described the formation of osteoclasts from cells of degenerate bone and cartilage, changes which have also been seen by one of us. Furthermore, in the articular cartilage of a rat, which was invaded by a methylcholanthrene-induced fibrosarcoma, the chondrocytes were greatly enlarged and granular, closely simulating many of the giant cells seen in this phalangeal tumour. Moreover, in view of the mutations of cell form which may be seen in active callus, it is not surprising that wide variation may occur in cell differentiation in mesenchymal tumours of a similar origin. From these considerations, the second alternative of an origin in pluripotential cells would seem preferable. There were no features in the present tumour to support the assertion of Foster (1947), who suggested a vascular origin for giant-cell synovioma.

As a corollary to King's morphological classification of synoviomata, one may perhaps borrow by analogy the classification applied by Meyerding and Jackson (1950) to giant-cell tumours of bone, with reference to the whole group of synovial tumours (the term "synovial" is here used in a descriptive rather than a histogenetic sense), namely benign, malignant (*a*) malignant *ab initio* and (*b*) showing delayed malignancy, which may possibly be associated with such factors as trauma, sepsis, surgical intervention, and/or irradiation.

Subdivision in this manner overlaps the classification suggested by Stevenson (1950), who separated giant-cell synovial tumours into two groups on the basis of presence or absence of concomitant general metabolic changes.

The more slowly growing benign synoviomata not infrequently show some atypical mesenchymal differentiation, often in the form of dense collagen (simulating osteoid), myxoid tissue, or occasionally islets of cartilage. The *in vitro* tissue culture methods applied to three synovial sarcomata by Murray, Stout, and Pogogeff (1944) yielded results interpreted as representing two main tissue forms, synovial and fibroblastic, but it was uncertain whether the latter may not have arisen from reactive, vascular, or other mesenchyme included in the tumour tissue taken for culture. Whatever may be the true explanation, the fibroblast element may at least be considered as having a potential ability to differentiate in many ways under a suitable stimulus.

With the disordered metabolism of more aggressive growth, as in malignancy, a wider stromal differentiation may be expected and appears as sarcoma, which morphologically may take any of the forms of

synovial sarcoma of the more usual classic pattern, reticulo-sarcoma, fibrosarcoma, osteogenic sarcoma, chondrosarcoma, malignant giant-cell tumour, or mixed tumours in which the various cell and stromal types are intermingled.

One may from this assertion then perhaps pose the question, If sarcoma is related to the existence of a preceding benign tumour, why the very marked disproportion of the malignant and benign forms of neoplasia in the digits where the former are very rare and the latter relatively common? One obvious feature with tumours of the hand and foot is the urgency of symptoms, which lead to early treatment. In other anatomical sites, the proportion of malignant forms of synovioma would be much higher, and more closely follow the group incidence of malignant forms of other mesenchymal tumours, e.g., giant-cell tumours of bone or chondroid growths of major bones.

Considered in this light, it becomes apparent that the histological picture of giant-cell synovioma may be shown by a mesenchymal growth potentially capable of further tissue differentiation in several directions. This parallels in most respects the behaviour of giant-cell bone tumours. The literature relating to the latter growths records acceptable examples of supervening or associated malignancy taking the form of fibrosarcoma, osteogenic sarcoma, and malignant giant-cell tumours of mainly osteolytic form. There are also reported cases of giant-cell bone tumours which have been malignant from the time of initial examination, although, in such cases (e.g., that reported by Cameron and Marsden, 1952), the history may be atypical in symptom sequence or timing, either for benign giant-cell tumour or primary sarcoma.

The most tenable view is surely that giant-cell tumours, whether of bone or otherwise, grade from the so-called "benign" to the obviously malignant; and that the features, both clinical and histological, which typify the latter may appear either at an early or late stage, and may possibly be provoked by the factors mentioned above.

While any attempted sub-division of a tumour type may add unwanted fuel to the conflagration which burns around the differentiation of benign and malignant forms, it yet may be of some practical value. The useful application of this would seem to be the rational basis for treatment and prognosis, founded upon careful consideration of at least five major features in each instance, viz., the tumour type, histological grading, site, stage, and potential ability for further aggressive development.

Summary

A detailed description is given of a malignant giant-cell synovioma of the phalanx apparently of intraosseous origin.

The structure of this tumour has been interpreted in the light of other closely related growths, and a histogenesis from pluripotential mesenchymal cells is suggested.

The relationship and similarity of behaviour of this tumour to other giant-cell mesenchymal growths is discussed.

The authors wish to record their thanks to Mr. G. S. Storrs for permission to publish this case. The personal examination of this tumour made by one of us forms part of a wider programme of investigation into the causation of giant-cell tumours and their relationship to osteogenic sarcoma. This more extensive research is generously supported by the British Empire Cancer Campaign. The reproductions of the radiographs and photomicrographs are the work of Mr. J. E. Hancock, of the Medical Research Laboratory, Department of Pathology, University of Bristol.

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WIDESPREAD SEROUS MEMBRANE INVOLVEMENT BY RHEUMATOID NODULES

BY

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The characteristic cutaneous rheumatoid nodule, described fully by Collins (1937) and Bennett, Zeller, and Bauer (1940), is composed of a central area of fibrinoid necrosis around which develops an encircling corona of closely packed mesenchymal cells, the great majority of which appear to be fibroblasts (Collins, 1937), and external to this is a zone of inflammatory tissue.

Lesions with similar histological features occur in the synovial membranes, and have been described involving serous membranes such as the pleura (Bennett *et al.*, 1940; Baggenstoss and Rosenberg, 1943; Gruenwald, 1948), and Raven, Weber, and Price (1948), in whose case the peritoneum was also involved. Rheumatoid lesions have been known for some time to involve the pericardium (Baggenstoss and Rosenberg, 1944; Feiring, 1945; Clark and Bauer, 1947, 1948; Raven *et al.*, 1948) the myocardium (Clark and Bauer, 1948), the ring and cusps of the mitral and aortic valves (Grzimek, 1932; Baggenstoss and Rosenberg, 1941; Bywaters, 1950; Sokoloff, 1953a), the aortic valve (Pirani and Bennett, 1951; Bauer, Clark, and Külka, 1951), and the tricuspid valve (Baggenstoss and Rosenberg, 1941; Gruenwald, 1948).

Ellman (1947) and Ellman and Ball (1948) have described interstitial involvement of the lung in rheumatoid disease.

It is therefore thought that the present case may be of interest as it illustrates the widespread clinical manifestations associated with rheumatoid lesions of the pericardium, myocardium, endocardium, all heart valves, sclera, visceral pleura, and dura mater. As far as can be ascertained involvement of the dura mater has not previously been recorded.

Case Report

J. S., a retired jeweller aged 63, was first seen in 1951 with a 17 years' history of rheumatoid disease affecting principally the knees, wrists, metacarpophalangeal joints, and ankles. In the last seven years of the illness the exacerbations of the joint swellings coincided with attacks of "bronchitis," dyspnoea, and purulent sputum. While these attacks lasted the joints were always swollen and painful; and remissions, with a fall in the erythrocyte sedimentation rate, occurred as the chest symptoms subsided.

Before admission the patient suffered increasing dyspnoea, loss of weight, swollen and painful joints, crops of subcutaneous nodules, and a painful left eye.

The patient was an emaciated pale man; he was apyrexial and orthopnoeic. His left eye showed a patch of episcleritis on the lateral corneal-scleral margin with a raised nodule in the centre. Painful nodules 1 to 2 cm. in diameter covered the external occipital protuberance, the left olecranon process, the ulnar borders of both forearms and the ischial tuberosities. The wrists, metacarpophalangeal joints, and the knees were very swollen.

The trachea was central and there was no finger clubbing. Clinical examination of the lungs showed dullness, and absent breath sounds over the left base with localized rales.

The jugular venous pressure was raised, the heart was slightly enlarged with the apex beat 4½ in. from the mid-sternal line. The sounds were normal; the blood pressure was 105/65 mm. of mercury. The pulse rate was 45 and an electrocardiogram showed 2:1 heart block with right axis deviation.

The liver and spleen were not palpable.

The cranial nerves were normal and no abnormality was noted in muscular power and sensation apart from the wasting secondary to the arthritis. The reflexes and plantar responses were normal.

Radiology of the chest confirmed the cardiac enlargement, and cardioscopy showed this to be confined to the right ventricle. An effusion was present at the left base.

Thoracentesis showed a clear, straw-coloured fluid in which no malignant cells were seen, and cultures were sterile. Guinea-pig inoculation for tuberculosis was negative.

Analysis of urine and renal function tests were normal.

The erythrocyte sedimentation rate fluctuated throughout his stay in hospital between 80 and 100 mm. per hour (Westergren). A haematological examination showed a mild hypochromic anaemia with a haemoglobin level between 10.6 and 11.3 g.%.

After a period of observation the patient was digitalized, and given "mersalyl" twice a week. This had no effect on the pleural effusion. It was then decided to try the effects of hormone therapy, and he received corti-

sone, 100 mg. daily, over a period of 32 days. On this regime the joint swellings and nodules regressed, the dyspnoea became less distressing, and he could sleep on two pillows.

The dose of cortisone was gradually reduced to 25 mg. daily, and discontinued on the seventy-first day. The pleural effusion, however, remained uninfluenced. There was no change in the serum electrolytes, and at no time was oedema or a rise of jugular venous pressure noted.

The patient returned to his home and attended as an out-patient at regular intervals. Four months after cessation of cortisone no change in physical signs was noted and the radiology of the joints remained the same.

During the last two months of his life the patient began to suffer from episodes of loss of consciousness preceded by what appeared to be Jacksonian fits. He died at home following such an episode in May, 1953.

Necropsy Findings

The body was that of an emaciated old man showing multiple subcutaneous nodules about the elbows, neck, occiput, and ischial tuberosities.

Each pleural cavity contained about 500 ml. of thin, watery, yellowish fluid. The parietal and visceral pleura were covered with large masses of fibrin giving them a shaggy appearance, which was more marked over the lower lobes. On the visceral pleura, where it was not covered with fibrin, occasional small yellow nodules up to 3 mm. in diameter were present.

The right lung weighed 620 g. and the left 475 g. There was a generalized chronic bronchitis with some mucopus in the bronchi. On section the lungs were emphysematous, but the striking feature was the presence of numerous spherical lesions, some quite small, others as large as 1 to 2 cm. in diameter, scattered in the peripheral and sub-pleural parts of the lungs; none was found in the central parts. These lesions had a soft, cheese-like centre surrounded by collapsed and thickened lung parenchyma. The pleura was thickened by layers of fibrin up to 2 cm. with an occasional abscess cavity in it.

The pericardial cavity was obliterated by dense fibrous tissue, and its two layers were torn apart with difficulty, revealing an abscess cavity filled with greenish pus in its posterior part.

The heart weighed 264 g. and showed a flabby, soft, atrophic myocardium. There was some dilatation of the right ventricle. On opening the organ many nodules up to 0.5 cm. in diameter were seen on the endocardium, especially that of the left auricle and ventricle close to the mitral and aortic valves; two similar nodules were present on one cusp of the tricuspid valve, and several on one cusp of the aortic and pulmonary valves and on both cusps of the mitral valve. Occasional similar lesions were found in the substance of the myocardium and also in the epicardium about the root of the aorta. There were no lesions in the interventricular septum.

Similar nodules were present in the intima of the ascending part of the aorta. A moderate degree of atheroma affected the abdominal part. No abnormality was noted in other large arteries examined.

The abdominal organs were normal apart from a small cortical infarct in the left kidney.

The only abnormality in the endocrine system was adenomatous hyperplasia of the left adrenal.

Nothing of note was seen in the lymphatic system.

The brain weighed 1,380 g. The dura mater was adherent to both cerebral hemispheres over the vertex, but was readily separated from the pia. In the subdural space two abscesses containing greenish pus approximately 2 × 1 cm. were present on each side of the falx. Minimal atheroma of the cerebral arteries was present but the brain itself was normal.

The subcutaneous nodules in the scalp and about the elbow varied from 1 to 2 cm. in diameter and had a soft necrotic centre.

The joints examined showed thickening and hypertrophy of the synovial membrane and erosion of the articular cartilage.

Pus taken from the various lesions grew only *Proteus vulgaris*, which was regarded as a post-mortem contaminant, because necropsy had been delayed for 24 hours and the body had not been refrigerated.

Histology

Tissues removed at necropsy were fixed in formol-saline and stained with haematoxylin and eosin. Selected tissues were stained with phosphotungstic acid haematoxylin, Weigert's fibrin stain, Lendrum's modification of Mallory's trichrome stain, periodic-acid-Schiff, and silver impregnation for reticulin fibrils. Gram's stain and Ziehl–Neelsen's stain for micro-organisms were also used.

FIG. 1.—Photomicrograph of rheumatoid lesion in dura mater. ×96.

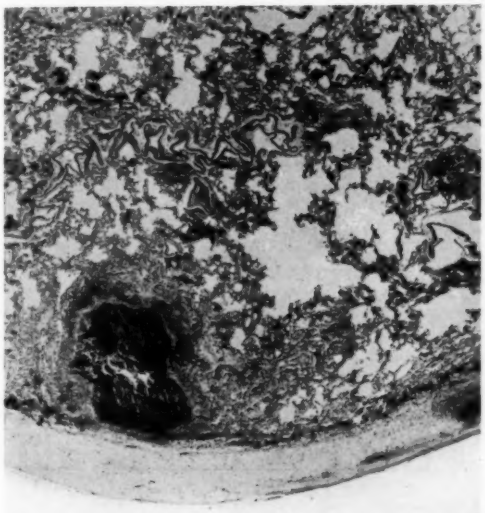

FIG. 2.—Photomicrograph of section from left lung showing thickened visceral pleura and rheumatoid lesion in adjacent lung. ×6.

In the spleen congestion and acute inflammation of the pulp with slight hyalinization of the Malphigian arterioles were observed.

The liver showed congestion and slight focal fatty change.

In the kidneys slight ischaemic fibrosis was seen.

A mediastinal lymph node revealed chronic inflammation.

Post-mortem autolysis and considerable adenomatous hyperplasia were present in the left adrenal.

In the dura mater (Fig. 1) generalized chronic inflammation of the cranial aspect was noted. The arachnoid surface of the dura was converted into granulation tissue in which were multiple rheumatoid lesions characterized by central necrosis and radially arranged elongated cells with pale nuclei and indistinct outlines. The central necrotic area was either amorphous and eosinophilic or showed pyknotic and fragmented nuclei, some of which resembled degenerating polymorphs. The radially arranged cells were surrounded by a zone of histiocytes, plasma cells, and lymphocytes.

The pleura (Fig. 2) was grossly thickened and showed much collagen moderately infiltrated with chronic inflammatory cells. Occasional rheumatoid lesions with degenerating cells in the central zone were present in the pleura and also in the lung adjacent to it. The alveoli near the lesion were collapsed, fibrosed, and showed

FIG. 3.—Photomicrograph of section of left auricular myocardium and endocardium showing two rheumatoid lesions in the endocardium. ×10.

FIG. 4.—Photomicrograph of section of left ventricle showing rheumatoid lesions in myocardium. ×10.

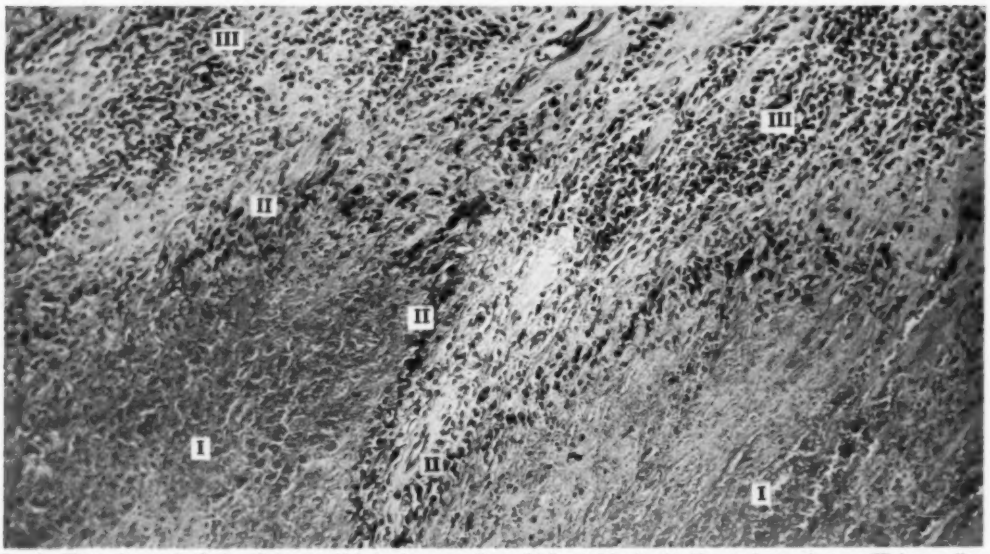

FIG. 5.—Photomicrograph of edge of rheumatoid lesion in myocardium of left ventricle showing (I) central necrotic zone, (II) intermediate zone of radially arranged elongated cell, (III) outer zone of chronic inflammatory cells. ×130.

some subacute inflammation. Elsewhere in the lower lobes the alveoli showed generalized collapse, fibrosis, and subacute inflammatory change.

The pericardium showed dense fibrosis and a diffuse sparse infiltration of chronic inflammatory cells. Sections prepared from the abscess cavity in the posterior part of the pericardium showed typical rheumatoid lesions.

In the myocardium and valves (Figs. 3, 4, and 5) the rheumatoid lesions were sharply demarcated from the normal structures. Where they abutted on the endocardium the latter was undamaged and no ulceration was observed. The myocardium showed generalized cloudy swelling and fragmentation with some interstitial oedema; no abnormality was noted in the connective tissue between the muscle fibres or in the small arterioles.

The typical appearance of the rheumatoid lesion in this case may be summarized as follows. Three zones may be distinguished (Fig. 5): (1) a central necrotic zone; (2) an intermediate zone of radially arranged elongated cells with pale nuclei and indistinct outlines; and (3) an outer zone of chronic inflammatory cells in which plasma cells are numerous.

The radially arranged cells of the intermediate zone show mitotic figures and giant cells are sometimes present. In the majority of lesions the central necrotic zone is composed of degenerating leucocytes, but an occasional one shows an intensely eosinophilic, structureless and slightly granular necrotic centre which does not stain with phosphotungstic acid haematoxylin, Weigert's fibrin stain; or Mallory's stain. Silver impregnation for reticulin fibres shows these to be interrupted at the edge of the lesion, and only an occasional fibril persists in the necrotic area (Fig. 6). The margin of the necrotic zone stains with the periodic-acid-Schiff technique.

The vasculature of the outer inflammatory zone was carefully examined by serial section, but no gross lesion

FIG. 6.—Photomicrograph of myocardium showing (I) interruption of reticulin fibrils at edge of the necrotic zone of the lesion and (II) persistence of an occasional fibril in the necrotic zone (Silver impregnation). ×80.

was seen apart from some intimal thickening of the smaller arterioles. No remnants of vessels were observed in the central necrotic zone.

Discussion

The clinical interest of this case centres in the symptoms and signs produced by the widely scattered rheumatoid lesions.

During the patient's final admission the pleural effusion and the 2 : 1 heart block dominated the picture. Whilst in hospital it was presumed, in view of the many subcutaneous nodules (tuberculosis, malignant disease, and cardiac failure having been excluded), that both the effusion and the heart block might be related to rheumatoid lesions on the pleura and close to the bundle of His. The lack of response of both of these conditions to hormonal therapy, in spite of regression of the subcutaneous nodules, is of some interest. It was surprising to find such gross cardiac involvement when the only clinical finding was heart block. A similar discrepancy was noted by Baggenstoss and Rosenberg (1941). In their series 14 cases showed gross cardiac lesions at necropsy, but only seven had clinical signs. Although epileptiform fits of Jacksonian type were observed at the latter part of the illness no cause for these was surmised, but the presence of rheumatoid lesions on the dura mater at necropsy suggests an obvious relationship.

The pleural effusion in this case is obviously related to the rheumatoid lesions on the pleura. It may be of interest in this respect that Robertson (1952) found four out of 58 pleural effusions in patients over the age of 40 to be due to " collagen diseases."

In this case many of the rheumatoid lesions showed pyknotic and fragmented nuclei in the central zone suggestive of degenerating polymorphs. This feature was also observed by Baggenstoss and Rosenberg (1944) and by Raven *et al.* (1948) and is to be regarded as a variant of the typical hyaline " fibrinoid " necrotic centre as described by Collins (1937) and Bennett *et al.* (1940).

It is probable that in this case the necrotic change in the lesions had progressed beyond the " fibrinoid " stage and complete necrosis had taken place ; this is evidenced by the relatively weak periodic acid Schiff reaction seen mainly in the marginal part of the necrotic zone of the lesions. The interruption of reticulin fibrils, as shown in Fig. 6, indicates that the necrotic lesion has developed in pre-formed collagen and not as an additional exudative lesion.

Although the necrobiotic lesion might be considered to have a superficial resemblance to tuberculosis the palisaded cellular zone is essentially different from its tuberculous counterpart, and in appropriately stained sections no tubercle bacilli or other organisms were found.

The aetiology of the rheumatoid lesions cannot be stated with certainty ; two opposing theses are current in the literature. The relationship between vascular changes and nodule formation was first emphasized by Von Glahn and Pappenheimer (1926) in rheumatic fever, and Sokoloff (1953b) considers that the subcutaneous rheumatoid nodule results from occlusive lesions in the subcutaneous vasculature. This view is supported by Bennett *et al.* (1940) who found remnants of pre-existing blood vessels in the central necrotic zone of such rheumatoid nodules, and they emphasize the frequency of vascular changes in the peripheral inflammatory zone and even suggest that the necrosis may be partly due to infarction.

On the other hand Collins (1937, 1949) states that the fibrinoid degeneration arises in the proliferating mesenchymal cells of the nodule and " spreads outwards, encroaching first on the newly proliferated tissue and then on older structures, such as blood vessels and mature collagen bundles," and in his Fig. 4 (1937) this is well illustrated.

While it is admitted that vascular lesions may be difficult to demonstrate careful examination of many sections of rheumatoid lesions from this case failed to reveal any appearance suggestive of antecedent vascular changes ; and in sections prepared from tissues not showing actual lesions no vascular changes were noted.

The observations made in this case do not illuminate the aetiology of these nodules, but the widespread distribution of the lesions emphasizes the protean nature of rheumatoid disease in its disseminated form.

Summary

A case of long-standing rheumatoid disease is described in which widespread rheumatoid lesions were associated with pleural effusions, heart block, and Jacksonian epilepsy. The histological appearances of the lesions in the visceral pleura, endocardium, pericardium, and dura mater resemble the well-known subcutaneous nodules of rheumatoid disease.

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THE DETERMINATION OF SERUM ACID AND ALKALINE PHOSPHATASE ACTIVITY WITH 4-AMINOANTIPYRINE (A.A.P.)

BY

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Phenolic compounds give red or purple colours with 4-aminoantipyrine (A.A.P.) in the presence of alkaline oxidizing agents (Emerson, 1943). The amino group of the A.A.P. condenses with the phenol to give a substance which is oxidized to a coloured quinone.

Gottlieb and Marsh (1946), using alkaline ferricyanide as the oxidant, devised a method for the estimation of certain phenolic fungicides with A.A.P. The procedure was modified by Grifols (1951) for the determination of phenol in the measurement of alkaline phosphatase activity in body fluids. This author made a careful study of the factors which control the development of the colour and his results showed good correlation with those obtained by the method of King (1951). Advantages claimed for the method were that the reagent did not react with proteins, thus obviating their precipitation during the estimation, and also the colour development was comparatively rapid. The use of A.A.P. as a reagent for phenol appeared to be of potential value especially if its use could be extended to the estimation of serum acid phosphatase activity. Considerable modification of the original Grifols procedure was found to be necessary in order to measure both alkaline and acid phosphatase activity in blood. The use of calibration curves, the stability of the colour, and the effect of the presence of serum on the colour development were also investigated. Results obtained with the proposed methods in a number of normal and pathological sera were compared with those obtained with King's methods.

Experimental

Reagents.—4-Aminoantipyrine hydrochloride (Hopkin & Williams, Ltd.) was used without further purification. Potassium ferricyanide was of AR standard. The sodium carbonate/bicarbonate buffer, citric acid/sodium citrate buffer, and diphenyl phosphate substrate solution were prepared according to the directions of King (1951).

Standard phenol solutions were prepared by diluting a solution containing 1 g. of pure crystalline phenol in 1 litre of water. The phenol content of this latter solution was estimated with N/10 bromine (British Pharmacopoeia, 1953).

The Estimation of Acid Phosphatase Activity.—In acid solutions, oxidizing agents convert A.A.P. to the deeply coloured "antipyrine red" (Emerson, 1938) so that A.A.P. and ferricyanide cannot be used to estimate phenol produced after incubation of diphenyl phosphate substrate and serum in an acid buffer at *p*H 4.9. In the original method of Grifols for alkaline phosphatase the *p*H of the final mixture after the addition of M-10 carbonate was 10.2. The corresponding mixture of serum and substrate after incubation with a citric acid buffer in the acid phosphatase method was at *p*H 4.9, and 1 ml. of 22.5% w/v aqueous Na_2CO_3 was found to be necessary to produce a final *p*H of 10.2. Table I shows the results obtained from a series of phenol solutions of concentrations ranging from 10 to 100 μg./ml. which were estimated by the following two procedures; the concentrations of A.A.P. and ferricyanide employed were those used by Grifols (1951).

Method A	Method B
2 ml. phenol solution	2 ml. phenol solution
2 „ alkaline buffer	2 „ acid buffer
1 „ M-10 sodium carbonate	1 „ 22·5% sodium carbonate
1 „ 0·06% A.A.P.	1 „ 0·06% A.A.P.
1 „ 0·24% potassium ferricyanide	1 „ 0·24% potassium ferricyanide

TABLE I
COMPARISON OF OPTICAL DENSITIES USING METHODS A AND B

Phenol (μg./ml.)	Method A	Method B
10	0·090	0·060
15	0·135	0·095
20	0·180	0·125
25	0·220	0·160
30	0·265	0·185
40	0·330	0·230
50	0·390	0·245
75	0·520	0·370
100	0·660	0·455

The mixtures were allowed to stand at room temperature for 15 minutes and the optical densities measured in ½ cm. cells in the Spekker absorptiometer using Ilford spectrum green filters No. 604.

The results show that, although the *p*H of the mixtures after the addition of the carbonate solutions were identical, the optical densities in method B were approximately 30% lower than those using method A. It therefore appeared that the citrate present in the acid buffer interfered in some way with the production of the colour. A similar result was observed when acid buffers containing acetate or phthalate were used. Gottlieb and Marsh (1946) reported that attempts to utilize alkaline borate and glycine buffer mixtures in their work on the estimation of phenolic fungicides were not successful because the colour development was decreased.

On the empirical reasoning that the buffer ions in some way reacted with the reagents, it was decided to increase the concentrations of A.A.P. and ferricyanide in methods A and B described above. Table II summarizes this series of experiments, a 40 μg./ml. phenol solution being used throughout.

TABLE II
EFFECT OF INCREASING REAGENT CONCENTRATIONS

A.A.P. (g./100 ml.)	Ferricyanide (g./100 ml.)	Optical Density	
		Method A	Method B
0·06	0·24	0·320	0·230
0·12	0·48	0·460	0·235
0·24	0·96	0·580	0·244
0·50	1·0	0·631	0·383
1·0	2·0	0·647	0·497
1·0	4·0	0·650	0·559
1·5	4·0	0·625	0·563
1·5	6·0	0·627	0·560
2·0	2·0	0·490	0·354
2·0	6·0	Turbid solutions	

The results show that there was some improvement in the optical densities obtained with increasing amounts of reagents. However, the agreement with the results obtained with the two methods was still unsatisfactory. One point which emerged from the work was that the original Grifols concentrations did not give full colour development and were inadequate for amounts of phenol corresponding to phosphatase values above 100 units. If excess phenol were present it acted as a reducing agent converting the red quinone to a colourless leuco compound. Fig. 1 illustrates this effect using increasing amounts of phenol and 0.06% A.A.P. and 0.24% ferricyanide.

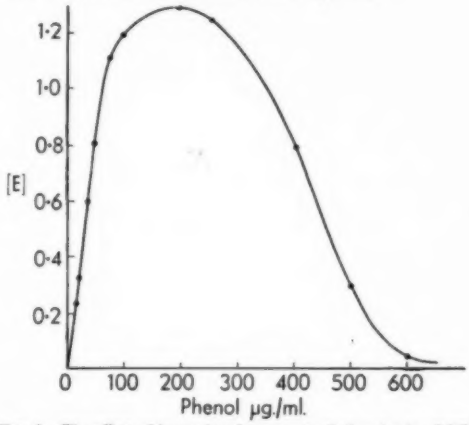

FIG. 1.—The effect of increasing the amount of phenol using 0.06% A.A.P. and 0.24% ferricyanide.

The only difference between the substances used in the alkaline and acid buffers and their subsequent treatment apart from the citric acid and citrate in the acid buffer was sodium bicarbonate. It was therefore decided to investigate the use of various carbonate/bicarbonate mixtures instead of 22.5% carbonate in method B. 4-Aminoantipyrine, 1.5 g./100 ml., and 4 g./100 ml. ferricyanide were used because the results in Table II indicated that these were the optimum concentrations of the reagents. Table III summarizes the results of these experiments using a 40 μg./ml. phenol solution.

The results indicated that both the concentrations and ratio of the sodium carbonate and sodium bicarbonate in the mixture were important factors. Although the addition of 1 ml. of a solution containing 7.5 g./100 ml. of sodium carbonate and sodium bicarbonate gave optimum results, the addition of 2 ml. of the 4 g./100 ml. concentrations was preferred because the former mixture deposited crystals on standing at room temperature.

TABLE III
EFFECT OF USING SODIUM CARBONATE/SODIUM BICARBONATE MIXTURES IN METHOD B*

Composition of Mixture (g./100 ml.)		Optical Density	
Na_2CO_3	$NaHCO_3$	Method A	Method B*
22·5	0	0·625	0·563
10	2·5	0·625	0·575
5	2·5	0·630	0·580
5	5	0·625	0·600
7·5	7·5	0·620	0·620
4†	4	0·545	0·550

* The concentrations of A.A.P. and ferricyanide were 1·5% and 4·0% respectively.
† 2 ml. of this mixture added in Method B and 2 ml. of M-10 sodium carbonate added in Method A.

Development and Stability of Final Colour.—Fig. 2 illustrates the increase in colour intensity with time of a phenol solution estimated by both acid and alkaline procedures. The colour with the alkaline method is fully developed in five minutes whereas with the acid method 15 minutes is necessary. The final colour is stable for at least two hours, but has diminished to approximately 90% of its maximum intensity in six hours.

Final Method.—The suggested procedures for acid and alkaline phosphatase estimations in serum are as follows:

Alkaline Phosphatase	Acid Phosphatase
Carbonate/bicarbonate buffer, 2·0 ml.	Citrate buffer, 2·0 ml.
Diphenyl phosphate solution, 2·0 ml.	Diphenyl phosphate solution, 2·0 ml.
Serum, 0·2 ml.	Serum, 0·2 ml.
Incubate at 37° C. for 15 min., then add	Incubate at 37° C. for 60 min., then add
M-10 carbonate, 2·0 ml.	4% carbonate / 4% bicarbonate (1:1) solution 2·0 ml.
1·5% A.A.P., 1·0 ml.	1·5% A.A.P., 1·0 ml.
4% ferricyanide, 1·0 ml.	4% ferricyanide, 1·0 ml.

The alkaline phosphatase estimations are allowed to stand at room temperature for five minutes, and the acid tubes for 15 minutes (cf. Fig. 2). The optical densities are measured in a Hilger Spekker absorptiometer using Ilford spectrum green filters No. 604. The optical densities are read against control solutions prepared as above except that the serum is added after the incubation.

Calibration Curves.—Calibration curves as shown in Fig. 3 may be constructed by substituting 2 ml. of phenol solution varying in concentration from 5 to 100 μg./ml. for the substrate solution. In the present method one phosphatase unit is equivalent to that of King and Armstrong, i.e., 2 μg. of phenol liberated from the substrate under the various incubation conditions; in the standard solutions used for the preparation of the calibration curves 1 μg./ml. of phenol equals 1 phosphatase unit. Each solution used for the calibration curves had 0.2 ml. serum added because the presence of serum decreases the optical density by 5 to 10% as shown in Table IV.

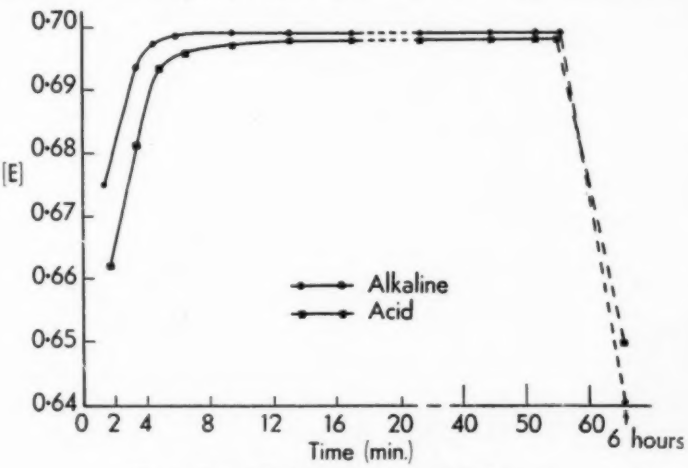

FIG. 2.—Rate of development and fading of final colour.

TABLE IV

EFFECT OF SERUM ON COLOUR DEVELOPMENT

Phenol Solution* (μg./ml.)	Optical Densities			
	Water	Amount of Serum Added		
		0·1 ml.	0·2 ml.	0·4 ml.
10	0·148	0·140	0·135	0·130
15	0·220	0·220	0·210	0·204
20	0·298	0·289	0·291	0·273
30	0·458	0·442	0·420	0·408
40	0·592	0·565	0·558	0·534
50	0·731	0·690	0·683	0·670

* The final volume of each solution was identical, the serum having been substituted for an equal volume of water.

Comparison of Results with those Obtained by King's Methods.—The results obtained with both the above methods and the methods of King (1951) for acid and alkaline phosphatase activities in a number of blood specimens are shown in Tables V and VI.

Conclusion

While the original method of Grifols was found to be adequate for the determination of alkaline phosphatase activity it could not be applied directly to the measurement of serum acid phosphatase activity because the anions present in the acid buffer solution interfered with the colour production. The procedure was modified by the addition of a bicarbonate/carbonate solution after the incubation and the use of much greater concentrations of A.A.P. and ferricyanide so that this interference due to the buffer anions was overcome. It has been possible to devise methods for the estimation of both types of phosphatase activity in serum using the same calibration curves. The presence of serum tends to decrease the colour intensity, and it is recommended that the calibration curves should be constructed with 0.2 ml. of serum in each estimation. This effect of serum was not studied by Grifols, who merely stated that A.A.P.

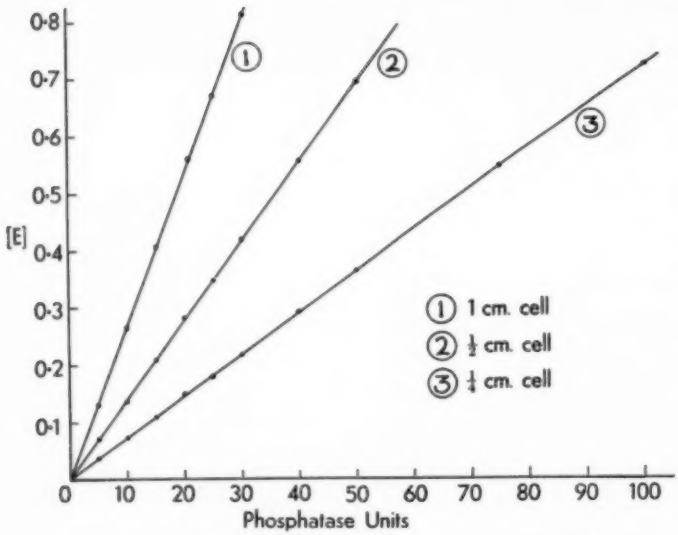

FIG. 3.—Calibration curves for both acid and alkaline phosphatase estimations using various sizes of optical cells.

TABLE V
COMPARISON OF METHODS FOR ESTIMATING SERUM ALKALINE PHOSPHATASE ACTIVITY

Case No.	Diagnosis	Phosphatase Activity (King-Armstrong Units)	
		King	Present Method
1	Normal adults	6	7
2		10	9
3		7	7
4		7	8
5	Normal child	20	19
6	Infective hepatitis	11	13
7		19	21
8		29	28
9		40	40
10	Obstructive jaundice	54	52
11		98	102
12		128	124
13	Carcinoma of pancreas—post-operative specimen	33	33
14	Paget's disease	30	30

TABLE VI
COMPARISON OF METHODS FOR ESTIMATING SERUM ACID PHOSPHATASE ACTIVITY

Case No.	Diagnosis	Phosphatase Activity (King-Armstrong Units)	
		King	Present Method
1	Normal subjects	2	1
2		2	3
3		3	3
4		2	2
5	Carcinoma of prostate with metastases	26	27
6		70	68
7		42	42
8		17	18

did not react with plasma proteins.

The results obtained with the present methods in a number of sera from normal persons and patients suffering from jaundice and carcinoma of the prostate have been compared with the results obtained by the method of King (1951). A good agreement between the results was observed as is shown in Tables V and VI. In our opinion the present methods have several advantages over those of King, notably in the speed and simplicity of manipulation. Serum proteins do not require precipitation, thus eliminating centrifugation and removal of a supernatant solution, and the final colour development does not involve an incubation. An estimation of serum alkaline phosphatase activity can be made in about 25 minutes, and the final colour is stable for several hours at room temperature.

Summary

The use of 4-aminoantipyrine (A.A.P.) for the measurement of phenol in the final stage of the estimation of serum phosphatase activity has been studied.

Methods have been devised for the determination of both serum acid and alkaline phosphatase activity using the same calibration curves.

Results with these methods have been compared with those obtained using the methods of King.

We wish to thank Miss E. Quilley for technical assistance and the Board of Governors of King's College Hospital, London, for a grant towards the cost of the work.

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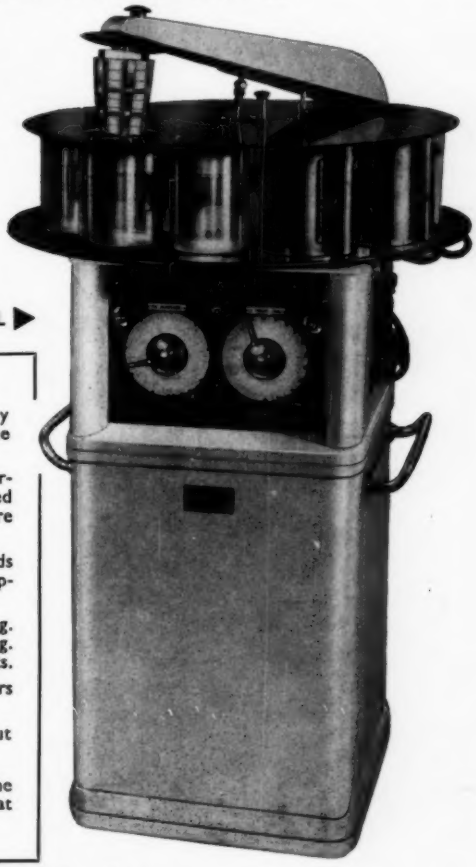

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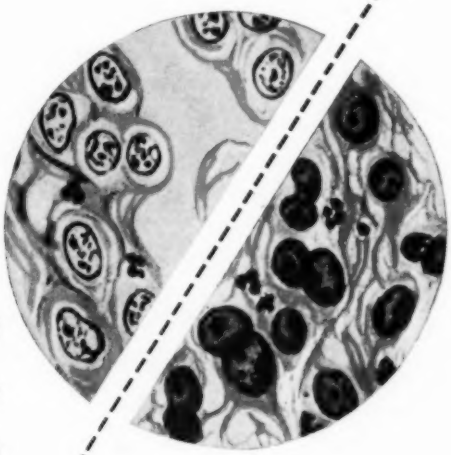

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J. clin. Path. (1954), 7, 249.

THE MODE OF ACTION OF PHENYLINDANEDIONE

BY

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Since the discovery (Soulier and Gueguen, 1947) that phenylindanedione (P.I.D.) was an anticoagulant which effectively altered the one-stage prothrombin time it has been used extensively in the treatment of cases of thrombosis (Blaustein, 1950; Blaustein, Shnayerson, and Wallach, 1953; Dennis, Coon, Hodgson, and Duff, 1952; Toohey, 1953). Clinically it appears to produce the same effect as dicoumarol, but its action is more rapid and less prolonged (Blaustein, 1950; Jaques, Gordon, and Lepp, 1950; Bjerkelund, 1950). Sufficient experience has not yet been accumulated to compare its efficacy with that of tromexan (ethyl-biscoumacetate), though the available data suggest that both are valuable in the treatment of thrombosis, leading to a reduction in thromboembolic complications. There is no clear evidence of the precise mode of action of phenylindanedione.

If a drug causes a prolongation of the clotting time in the one-stage prothrombin test this does not necessarily mean that prothrombin itself is involved. Confusion has arisen because some workers have made this assumption on the basis of the one-stage test, and as a result it is usually stated that P.I.D. depresses prothrombin production by the liver. It is now accepted that at least three factors influence the clotting time in this test, viz., factor V, factor VII, and prothrombin itself. Although the test, which was originally designed (Quick, Stanley-Brown, and Bancroft, 1935) to measure prothrombin as such, remains a satisfactory one for routine control of anticoagulant therapy, it is not a satisfactory research or diagnostic method because it does not distinguish between deficiencies of the three factors.

Owren (1951) and Owren and Aas (1951) stated that both prothrombin and proconvertin (factor VII) were decreased by phenylindanedione. Bjerkelund (1950) found that phenylindanedione depressed prothrombin in man, but the method he used (Owren, 1949) for estimating prothrombin makes no distinction between prothrombin and factor VII, being a measure of both. The same criticism applies to the experiments of Molho (1952) in rabbits: he reported that the main effect was on prothrombin. Richards (quoted by Jaques, 1950) and Seegers (quoted by Jaques, 1950) both found a marked fall in prothrombin in dogs given phenylindanedione, with a slight fall in factor V (globulin). Once again, however, it would appear that "prothrombin" as described by these workers includes both prothrombin and factor VII, so that either or both could be affected by the administration of phenylindanedione.

In order to determine which of the factors is principally affected by phenylindanedione the following methods were used.

Preparations

Factor V.—Normal citrated plasma was treated with $Al(OH)_3$ as described by Biggs, Douglas, and Macfarlane (1953). Aluminium hydroxide Cα, 0.1–0.4 ml., prepared by the method of Bertho and Grassman (1938), was added to 2 ml. of citrated plasma, and the mixture incubated at 37° C. for 15 minutes. The $Al(OH)_3$ was then removed by centrifuging, and the one-stage prothrombin test done on the supernatant plasma to ensure that the clotting time was longer than one minute. By this means, prothrombin and factor VII are removed by adsorption, leaving factor V. (This preparation also contains antihaemophilic globulin, and is used as a source of both factor V and antihaemophilic globulin in the blood thromboplastin generation test.)

Factor VII.—Normal blood was clotted in a glass tube containing three glass beads and incubated at 37° C. for one hour to promote maximum prothrombin conversion. The serum was then used as a source of factor VII.

Thromboplastin.—This was prepared from fresh human brain by the method described by Biggs and Macfarlane (1953a), and with it normal plasma gave a one-stage clotting time (Quick's test) of 11 to 14 seconds.

Calcium.—M-40 calcium chloride solution was used.

P.I.D. Plasma.—This term refers to plasma taken from a patient receiving phenylindanedione. Addition of P.I.D. to plasma *in vitro* has no effect.

Diluting Fluid.—Where tests were done with diluted plasma, normal saline was used as the diluting fluid.

Dosage of Phenylindanedione.—The dose was 100 mg. given twice on the first day, and thereafter 50–150 mg. daily in two doses, according to the response.

Determinations of Clotting Defect in P.I.D. Plasma

(1) **Quick's One-stage Test.**—When the one-stage test of Quick *et al.* (1935) gives a prolonged clotting time with a test plasma, the addition to the test plasma of a 10% concentration of the missing factor (factor V, factor VII, or prothrombin) results in a significant shortening of the clotting time towards that of normal plasma (Biggs and Macfarlane, 1935b ; Biggs and Douglas, 1953a). In our experiments, Quick's test was done on the plasmas of 38 patients under treatment with phenylindanedione, with prolongation of the " prothrombin " times (Table I). The test was then repeated using as test plasma, first, a mixture of 90% P.I.D. plasma with 10% factor V preparation ; secondly, as test plasma, a mixture of 90% P.I.D. plasma with 10% factor VII preparation ; thirdly, as test plasma, a mixture of 90% P.I.D. plasma with 10% normal plasma. This last mixture contains all three factors, including prothrombin, and if deficiency in prothrombin were responsible for the prolonged clotting time, then a greater shortening of the time would be expected from the addition of normal plasma to the test plasma than from the addition of the serum preparation, which contains only factor VII.

As shown in Table I, the greatest shortening of the clotting time in all cases results from the addition of 10% factor VII preparation—often, indeed, the correction is complete, the clotting time reaching that of normal plasma. The clotting time is corrected to a less extent by the admixture of normal plasma, and not at all by the admixture of factor V. This suggests that P.I.D. has no action on factor V, and that it acts on factor VII rather than on prothrombin.

In the first 10 cases of the series, moreover, 10% of tromexan plasma and 10% of tromexan serum were added to the P.I.D. plasma, and the one-stage test repeated. No significant reduction of

TABLE I

RESULTS OF EXPERIMENTS WITH PLASMA FROM PATIENTS RECEIVING PHENYLINDANEDIONE

Case No.	Normal Plasma Alone	P.I.D. Plasma Alone	90% P.I.D. + 10% Factor V Preparation	90% P.I.D. + 10% Factor VII Preparation	90% P.I.D. + 10% Normal Plasma
1	11	20	20	11	14
2	12	36	36	17	20
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
37	13	23	24	14	19
38	10	15	15	10	13
Mean values	12	26	26	13	19

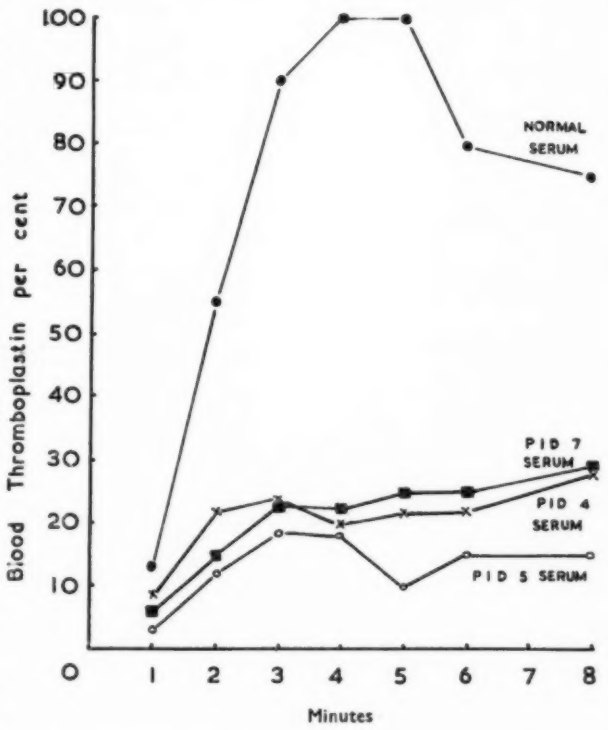

FIG. 1.—The curves show thromboplastin generated from normal $Al(OH)_3$-treated plasma and platelets using normal serum (upper curve), and from the same mixture using three P.I.D. sera (lower curves). All of 25 P.I.D. sera treated gave similar curves. 100% thromboplastin represents clotting time in substrate plasma of 10 secs.

the clotting time resulted in any case. As plasma from tromexan-treated patients lacks factor VII (Hunter and Tudhope, 1953), this is further evidence that the defect in P.I.D. plasma is also in factor VII.

(2) **Estimation of Prothrombin in P.I.D. Plasma.**—In 10 cases of the series the prothrombin content of P.I.D. plasma was measured by the two-stage area method (Biggs and Douglas, 1953a). The results are given in Table II, together with the

TABLE II

COMPARISON OF "PROTHROMBIN ACTIVITY" OF P.I.D. PLASMA WITH PROTHROMBIN BY TWO METHODS

Case No.	One-stage Test			2-stage Test
	Clotting Time Normal Plasma (Secs.)	Clotting Time P.I.D. Plasma (Secs.)	"Prothrombin Activity" P.I.D. Plasma (% of Normal)	P.I.D. Plasma Prothrombin (% of Normal)
29	14	45	10	28
30	14	29	16	92
31	11	20	25	47
32	11	15·5	35	61
33	11	14	43	70
34	11	24	18	32
35	11	17	30	69
36	11	20	25	41
37	13	23	20	35
38	10	15	33	38

corresponding one-stage "prothrombin" times and the percentage of "prothrombin activity" they represent on a saline dilution curve. In no case did the prothrombin, measured by the two-stage area method, fall below 28% of normal, with a range of 28% to 92%. According to Biggs and Douglas (1953a) reduction in prothrombin has little or no effect on the one-stage test until a level of 10% of normal is reached. If this is so, the lengthening of the one-stage "prothrombin" time when P.I.D. is administered in therapeutic doses may be attributed entirely to a reduction in factor VII.

(3) **Blood Thromboplastin Generation Test.**—In order to confirm that a clotting factor normally present in serum is lacking in patients treated with phenylindanedione, the blood thromboplastin generation test (Biggs *et al.*, 1953; Biggs and Douglas, 1953b) was used. This test demonstrates the formation of potent thromboplastin from a mixture of blood clotting factors, viz., platelet suspension, antihaemophilic globulin, factor V, factor VII, Christmas factor, and calcium. Normal serum is used as a source of factor VII and Christmas factor. This test was done using normal serum and also serum from 25 of the P.I.D.-treated patients, all the other factors being present in optimal amounts. In every case, thromboplastin generation with P.I.D. serum was defective (Fig. 1). The deficiency is not in Christmas factor, as lack of this factor does not prolong the clotting time in Quick's test, and is therefore probably in factor VII.

Summary

Phenylindanedione (P.I.D.) administration results in a reduction of both prothrombin and factor VII. With therapeutic doses the prothrombin deficiency is not of sufficient degree to influence coagulation and therefore the principal and important site of action of P.I.D. is factor VII. We have demonstrated that blood thromboplastin generation is impaired when P.I.D. serum is used as a source of factor VII. It is probable that the clinical results with phenylindanedione and the coumarin type anticoagulants are due to this and not to the effect on prothrombin.

We are indebted to Dr. Ann C. Donald for assistance in carrying out the prothrombin estimations, and Mr. T. Black for technical assistance. Part of the expenses of this research were defrayed from a grant by the Secretary of State for Scotland.

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J. clin. Path. (1954), 7, 252.

STUDIES OF THE EXCRETION OF POLYVINYLPYRROLIDONE BY THE NORMAL HUMAN KIDNEY*

BY

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In developing any plasma substitute the problem of finding a high molecular weight compound which can temporarily take the place of the plasma proteins is the major one, since the electrolyte composition involves no fundamental difficulties. The function of this high molecular weight compound is to exert an osmotic pressure across the blood capillary walls and, hence, assist in controlling the fluid balance in patients in whom there has been a loss of plasma protein. Provided any given polymer is pharmacologically inert and shows no toxicity either immediate, due to allergic reactions, or long-term, due to permanent storage of the foreign body in the tissue, its value in a plasma substitute will depend largely upon the persistence of the osmotic pressure which it exerts across the capillary wall. This will depend upon the rate of elimination of the polymer from the body and the rate at which it passes through the capillary walls into the tissue fluid. The elimination mechanisms will, in general, involve excretion of unchanged polymer by the kidney or by other means, and its degradation to smaller molecules. The passage of polymer molecules through both the kidney and the capillary wall will depend upon the shape, structure, charge, and molecular weight of the molecules present. The importance of molecular weight in selecting a polymer for use in a plasma substitute has recently been emphasized by Boyd, Fletcher, and Ratcliffe (1953), but not enough attention appears to be given to the effect that the shape and structure of a molecule can have on its passage through a membrane. Consequently, the molecular weight most suitable for use in a plasma substitute will vary with the type of polymer. The molecular weight distribution of a polymer does not necessarily remain constant after its infusion into the body, since it is unlikely that all the elimination mechanisms will be independent of molecular weight. For a complete understanding of the behaviour in the body of any polymer, knowledge of the variation of its molecular weight distribution with time is important. In all published work on the fate in the body of polymers, which are normally foreign to the body, no attempts appear to have been made to determine such changes in molecular weight distribution. In the present paper some of the changes in molecular weight distribution which occur in the case of the particular synthetic polymer, polyvinylpyrrolidone (P.V.P.), used in these studies are described.

Ideally, after an infusion of a plasma substitute, the polymer would be eliminated from the body at such a rate that the osmotic pressure due to the polymeric component decreased as the osmotic pressure due to the plasma proteins returned to normal as the deficiency in these was made good. An exact balance would, in practice, be impossible to achieve, but a rational approach to the problem of selecting the most suitable molecular weight range of any particular polymer is based on a knowledge of the time taken for the restoration to normal, after sudden depletion, of the plasma proteins in human subjects. Unfortunately, there appears to be insufficient data available at present on the rate of regeneration of plasma proteins under conditions similar to those when an infusion of a plasma substitute might be given. Most information on the regeneration of plasma proteins in human subjects is for cases of malnutrition. Wallace and Sharpey-Schafer (1941) have shown in human subjects not suffering from shock that regeneration of the plasma proteins was virtually complete in three to 90 hours, but no similar studies have been made on human subjects who are in a severely shocked condition due to loss of blood. Ebert, Stead, Warren, and Watts (1942) have shown that in dogs, severely shocked after considerable loss of blood which was replaced by an equal volume of isotonic saline, regeneration of proteins

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This is the second of a series of papers, the first of which was published in the *Journal of Polymer Science*, 1954, **12**, 611.

was extremely slow. No reason was given for this slow regeneration. It is not known whether the presence of a synthetic colloid can affect the regeneration of the plasma proteins through its property of maintaining the circulating volume.

In the absence of such information as would allow a theoretical approach to the development of a plasma substitute, it is of value to determine the behaviour in the body of those plasma substitutes which are clinically acceptable. Such a plasma substitute is "plasmosan," which was first described by Thrower and Campbell (1951). The clinical value of this material has been shown by Arden, Mandow, and Stoneham (1951), by Haler, Clift, Jameson, and Swinton (1953), and by Russell-Stoneham (1953). The present paper describes renal excretion studies of the colloidal component, P.V.P., from human subjects after infusion of "plasmosan." This polymer, originally examined for use in a plasma substitute by Hecht and Weese (1943), has since been further developed in France and in this country. Since much of the published work on this type of plasma substitute relates to P.V.P. of inadequately defined molecular weight range, conflicting results have been reported, and it must be emphasized that the results described in this paper apply to only one grade of P.V.P. of definite molecular weight distribution. A preliminary account of the present studies has already been given by Campbell (1953).

Experimental

At the hospital each patient, selected by the medical staff as being free from any kidney disease, was infused with up to one bottle (540 ml.) of "plasmosan." The time of beginning and ending the infusion was recorded, together with the volume of "plasmosan" unused. One 24-hour sample of urine was collected up to the time of the infusion. After the infusion individual samples of urine were collected during an initial period of about two days and then 24-hour samples for at least another nine days. In some surgical cases dressings which might contain P.V.P. from the open wounds were retained. In our laboratories all samples of urine were initially examined by the tests given below and then the P.V.P. was extracted. The molecular weight distributions of the extracted samples were determined. The wound dressings were also examined.

Details of Patients and Treatment.—Table I gives details of each patient together with notes on the loss of P.V.P. by various means. Mr. G. P. Arden, in charge of all the surgical cases, stated that there was no excessive bleeding in any case. The actual quantity of blood lost in the operation cannot, however, be stated.

Plasmosan from the same batch was used in all infusions. The composition of "plasmosan" has previously been given by Thrower and Campbell (1951). The molecular weight distribution curve for the P.V.P. used in this batch is given in Figs. 4 and 5, and had not changed during storage since the preparation of the "plasmosan."

Experimental Methods.—The clarity, specific gravity (by hydrometer), and *p*H (by glass electrode) of each sample of urine were noted. Albumin was tested for qualitativity by the sulphosalicylic acid test and for reducing sugar by Benedict's test. Creatinine was determined by the Jaffe method, the resulting intensity of colour being measured on an EEL colorimeter using a No. 624 filter. Details of the latter three tests have been given by Hawk, Oser, and Summerson (1947). The P.V.P. concentration was determined colorimetrically by the iodine method as described by Campbell and Hunter (1953) and by Campbell, Kane, and Ottewill (1954a). An EEL colorimeter with No. 623 filter was used to measure the intensity of colour. From the P.V.P. concentration, and the volume of the urine sample, the weight of P.V.P. present was obtained.

A representative quantity of the P.V.P. in each sample of urine was extracted by the following method. To the urine was added 22% by weight of exsiccated sodium

TABLE I
DETAILS OF PATIENTS

Patient Serial No.	Sex	Type of Case	Age	Diagnosis or Operation	Kidney Function from Clinical Evidence	Known Loss of P.V.P.
3	M	Medical	23	Suspected mildly active tuberculous pleural effusion	Normal	None
13	F	"	58	Duodenal ulcer	"	"
16	M	"	26	Pneumonia resolved before P.V.P. infused	"	D
17	M	"	31	Abdominal investigation—N.A.D.	"	None
18	M	"	61	Gastric ulcer	"	B, C, E
4	F	Surgical	73	Fractured left femur (Smith-Petersen pin)	"	E
5	F	"	54	Bilateral hallux valgus (Keller's)	"	B, E
6	M	"	18	Removal of exostosis of first metatarsal	"	E
7	F	"	46	Bilateral hallux valgus (Keller's)	"	C, E
8	F	"	53	" " " "	"	B, C, E
9	F	"	50	" " " "	"	B, E, A
10	F	"	39	" " " "	"	B, E
11	F	"	33	" " " "	"	C, E, A
12	F	"	21	Bilateral Keller's operation and spike arthrodesis	"	E
15	F	"	47	Right hallux valgus (Keller's)	"	

A = loss by menstruation, B = loss of urine by hospital, C = incontinence and/or bowels opening, D = loss of urine in laboratory, E = loss by bleeding into wound dressing.

sulphate, which was dissolved by heating. The solution was cooled to 25–30° C. and the P.V.P. was thrown out of solution as an insoluble gel. The solution was extracted three times with one-quarter its volume of chloroform. The separating funnel was shaken vigorously for five minutes for each extraction. Any gel formed was cleared on a centrifuge, and the "skin" formed at the interface was extracted with a little more chloroform to remove any adsorbed P.V.P. The chloroform extracts were combined and, if opalescent, were cleared by the addition of a small quantity of methanol. This had the effect of converting the two-phase system chloroform-water into a single phase, and sodium sulphate dissolved in the aqueous phase was precipitated. The clear liquid was then filtered and evaporated to dryness on a steam bath. The solid residue was freed from traces of chloroform by heating on a steam bath at reduced pressure (filter pump) and dissolved in an appropriate volume of water to give an aqueous solution which was then suitable for the determination of the molecular weight distribution. Experiments, in which P.V.P. of known molecular weight distribution was added to urine, have shown that there was no detectable difference between the dissolved and the extracted P.V.P. Distributions of molecular weight were determined by the turbidimetric method as described by Campbell, Kane, and Ottewill (1954b).

Each wound dressing was extracted with boiling water. After filtering, the aqueous solutions were concentrated by vacuum distillation and clarified in a centrifuge. The solutions were then examined by the iodine method for the presence of P.V.P. Attempts were made to determine molecular weight distribution curves. A wound dressing from a patient who had not received "plasmosan" was similarly examined.

Methods of Calculation.—Since the actual period of the infusion was at least one hour, zero time for calculating the rate of excretion of P.V.P. has been taken as the arithmetic mean of the beginning and end of this period. This introduces an indeterminate error when a comparison is made of the excretion by different patients at times less than 10 hours after infusion, but this error is negligible for longer periods.

The uncorrected weight of P.V.P. present in any sample of urine was obtained from the iodine estimation without allowance for variations in sensitivity of the method with molecular weight. When a correction was made for this, using the peak molecular weight of the distribution curve, the weight of P.V.P. present is referred to as the corrected weight. The correction factors used are given by Campbell, Kane, and Ottewill (1954a). This may introduce a small error, since the true correction factor, which cannot at present be determined, will depend upon the distribution of molecular weights rather than on the peak molecular weight.

In comparing the molecular weight distributions of the infused and total excreted P.V.P., it is convenient to consider the fate in each patient of 1 g. of P.V.P. which is completely characteristic of all the P.V.P. infused. Of each gramme infused, the fate of R/100 g. is known where R is the percentage total weight excreted. Therefore, molecular weight distribution curves for 1 g. of infused and R/100 g. of total excreted P.V.P. are compared. This latter is obtained by summing the molecular weight distribution curves for r/100 g. of each successive sample of excreted P.V.P. (of known percentage weight r) where $\Sigma r = R$.

Experimental Results

A complete picture of the renal excretion of P.V.P. can be obtained only from those cases in which there was negligible loss of P.V.P. (see Table I). The data from those patients in which indeterminate losses of P.V.P. did occur can, how-

TABLE II

EXPERIMENTAL DATA FOR PATIENT NO. 16 AFTER INFUSION OF 16·5 G. P.V.P.

Sample Number	Determination of P.V.P. in Urine						Laboratory Tests on Urine					
	Time from Infusion (hours)	Sample Volume (ml.)	Peak Molecular Weight $\times 10^{-3}$	Weight Factor	Uncorrected Weight of P.V.P. (g.)	Corrected Weight of P.V.P. (g.)	Clarity	Specific Gravity 15° C.	pH	Albumin	Sugar	Creatinine (g.)
16/1	Pre-infusion	1,090			0	0	Normal	1·013	6·1	—	—	1·31
16/2	0·5	475	10·5	1·48	4·89	7·24	,,	1·014	7·0	—	—	0·22
16/3	9	435	20	1·08	4·35	4·70	,,	1·015	6·0	—	—	0·64
16/4	18·5	330	21	1·08	0·99	1·07	,,	1·024	5·8	—	—	0·71
16/5	20·5	220	21·5	1·07	0·20	0·21	,,	1·012	6·6	—	—	0·10
16/6	21·5	360	25	1·05	0·19	0·20	,,	1·007	6·9	—	—	0·12
16/7	23·5	540	26	1·04	0·21	0·22	,,	1·015	7·0	—	—	0·15
16/8	45	1,800	25·5	1·04	1·14	1·19	,,	1·007	5·9	—	—	1·39
16/9	69	2,250	30·5	1·00	0·93	0·93	,,	1·011	6·5	—	—	1·91
16/10	93	2,034	33·5	1·00	0·51	0·51	,,	1·012	6·1	—	—	1·77
16/11	117	750	37	1·00	0·33	0·33	Highly turbid	1·023	5·8	—	++	1·77
16/12	141	1,935	34	1·00	0·23	0·23	Normal	1·012	6·1	—	—	1·82
16/13	165	1,660	36	1·00	0·14	0·14	,,	1·012	6·4	—	—	1·68
16/14	189	2,040	39	1·00	0·13	0·13	,,	1·015	6·0	—	—	1·77
16/15	213	2,710	40·5	1·00	0·11	0·11	,,	1·008	6·5	—	—	1·71
16/16	237	1,770	39	1·00	0·08	0·08	,,	1·008	6·5	—	—	1·50
16/17	261	1,340	47	1·00	0·02	0·02	,,	1·017	5·9	—	—	1·77
16/18	285	2,420	38	1·00	0·03	0·03	,,	1·009	6·3	—	—	1·79
16/19	309	2,605	49	1·00	0·02	0·02	,,	1·009	6·4	—	—	1·80
16/20	333	1,270	59	1·00	0·02	0·02	,,	1·013	6·2	—	—	1·46

ever, be used to support some of the main conclusions. A set of data for one typical medical patient is given in Table II.

Pattern of Excretion.—A typical set of molecular weight distribution curves for P.V.P. recovered from successive samples of urine passed by patient 16 is given in Fig. 1. The molecular weight distribution curves for all the samples of recovered P.V.P. showed the characteristics typical of fractionated material, a narrower molecular weight range and sharper peak than with the original unfractionated polymer. The curves, very sharp and narrow on the first day, gradually broadened as the post-infusion period extended. The increase in peak molecular weight with time after the infusion is illustrated in Fig. 2. Towards the end of the excretion study on any particular patient, about 150 hours after infusion, the difference between successive molecular weight distribution curves was less than the experimental error, which became large in this period due to the extremely small amount of P.V.P. present in the urine. The data for the cases which were incomplete through loss of urine or other causes were not in disagreement with this general picture.

Rate of Excretion.—Fig. 3 shows the variation of percentage weight excreted, with time after the infusion, for four medical and three surgical cases. The excretion rate, high during the first 24 hours after infusion, decreased to a small value after about 100 hours. Excretion did continue at this small, but measurable, rate for 300 to 600 hours for different patients. The rapid excretion immediately after infusion is illustrated by Table III, which includes data for all patients. The variation between different patients due to errors already dis-

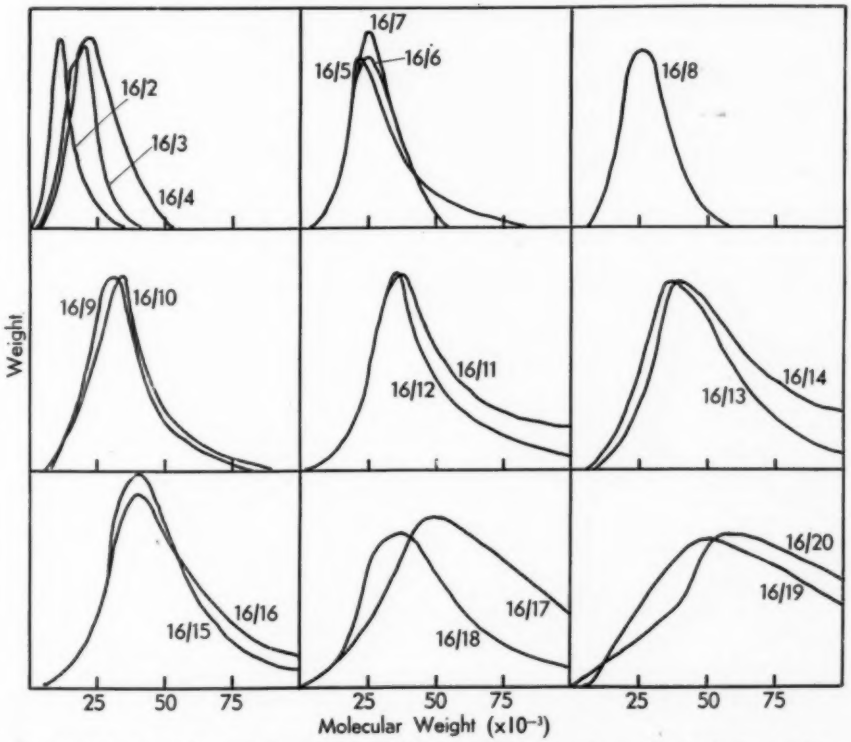

FIG. 1.—Molecular weight distribution curves for P.V.P. recovered from successive samples of urine from patient 16 (see Table II). These curves are not normalized.

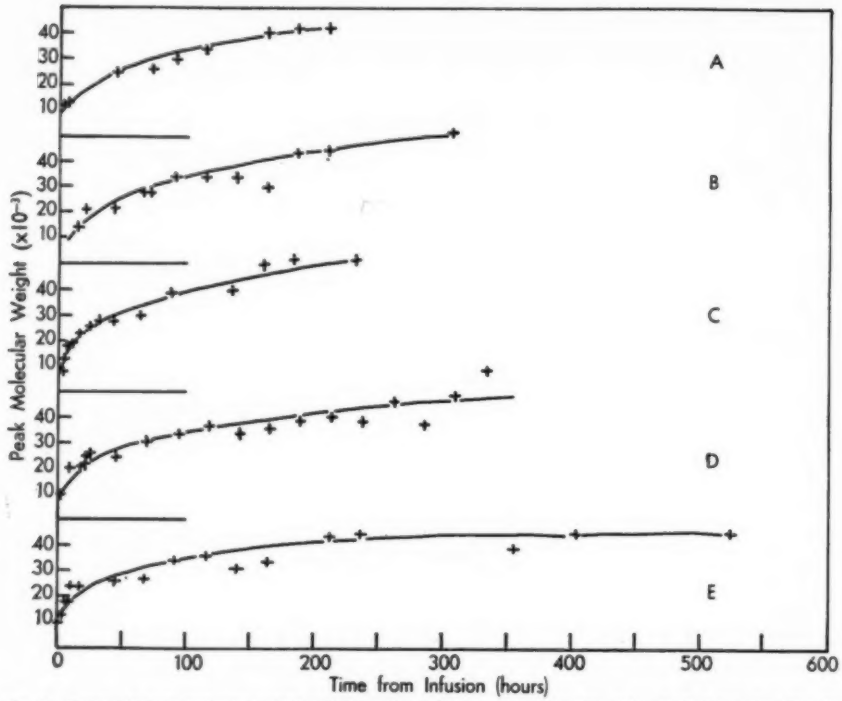

FIG. 2.—Increase in peak molecular weight with time after infusion. A is patient No. 9 (surgical), B is patient No. 12 (surgical), C is patient No. 3 (medical), D is patient No. 16 (medical), E is patient No. 13 (medical).

TABLE III
PERCENTAGE WEIGHT OF P.V.P. EXCRETED DURING FIRST 48 HOURS AFTER INFUSION

Type of Case	Patient No.	% Weight Excreted at 12 Hours		% Weight Excreted at 24 Hours		% Weight Excreted at 48 Hours	
		Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Medical	3	44	56	55	66	64	75
	13	62	75	70	83	75	84
	16	58	75	66	83	73	92
	17 (a)	66	—	73	—	81	—
	18	58	81	65	89	73	96
Surgical	4 (b)	—	—	—	—	—	—
	5	50	56	56	73	75	79
	6	12	14	22	25	35	40
	7 (c)	1	1	13	17	48	52
	8	12	15	22	27	32	38
	9	17	19	20	22	23	25
	10 (b)	—	—	—	—	—	—
	11	68	88	76	92	82	98
	12	32	40	39	47	44	52
	15	34	49	53	62	69	75

(a) Corrected weights are not available. (b) Data have not been calculated due to considerable loss of urine in this period. (c) The low values at 12 and 24 hours are probably due to retention of urine over this period.

cussed on page 254, in the times calculated for each sample of urine is small at 12, 24, and 48 hours after infusion.

An indication of the quantity of P.V.P. excreted over shorter post-infusion periods can be obtained (Table IV). Samples of urine, collected from two patients (Nos. 13 and 16) within 15 minutes of the end of the infusion, contain the P.V.P. actually

TABLE IV
EXCRETION OF P.V.P. DURING AND IMMEDIATELY AFTER INFUSION

Patient No.	Infusion		Time of Collection of Urine Samples	% Weight of P.V.P. Recovered	
	Time	Duration (hours)		Uncorrected	Corrected
13	10·45 to 11·45	1·00	11·50	3·5	5·2
16	10·05 to 12·20	2·25	12·34	29·8	44·1

excreted by the kidney during the infusion and for a short period afterwards. These data also indicate an immediate and rapid excretion of P.V.P.

Total Recovery of P.V.P.—The total recoveries of P.V.P. from all patients are given in Table V.

Renal Threshold Molecular Weight.—In all the patients studied, P.V.P. with molecular weights over the whole range 0 to more than 100,000 was recovered from the urine. If there is a threshold

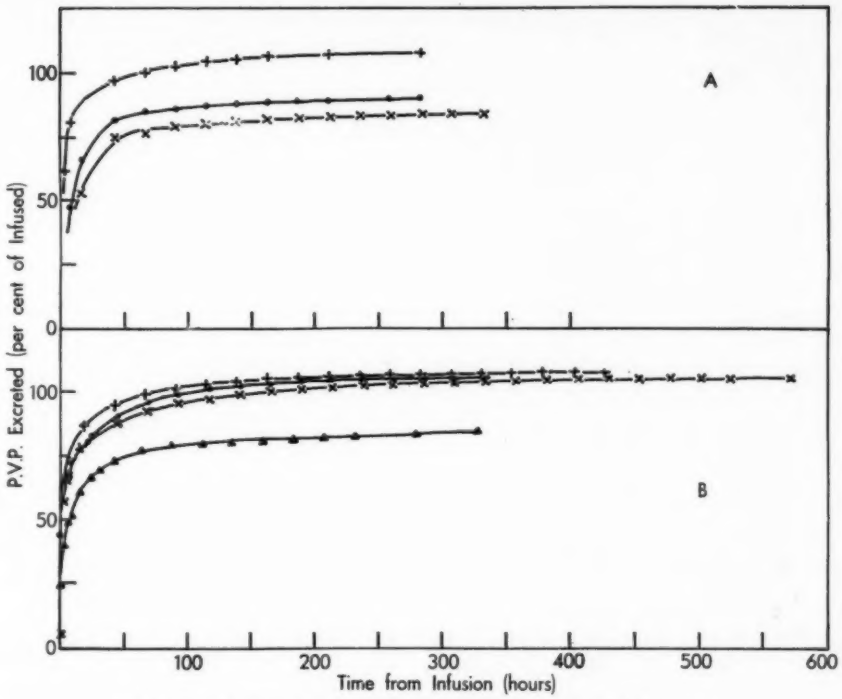

FIG. 3.—The variation, with time, of percentage weight of P.V.P. recovered from urine.
A + Patient No. 11 (surgical) B + Patient No. 18 (medical)
● Patient No. 5 (surgical) ● Patient No. 16 (medical)
× Patient No. 15 (surgical) × Patient No. 13 (medical)
▲ Patient No. 3 (medical)

TABLE V

TOTAL RECOVERY OF P.V.P. FROM URINE OF PATIENTS

Type of Case	Patient Number	Known Loss of P.V.P. other than by Bleeding from Wound	% Weight Recovered from Urine	
			Uncorrected	Corrected
Medical	3	None	70·3	88·0
	13	"	92·8	106·2
	16	"	87·9	105·3
	17	"	91·3	—*
	18	"	86·0	107·4
Surgical	4	Some	13·8	14·1
	5	None	81·5	89·8
	6	Some	42·4	46·6
	7	None	53·9	59·0
	8	Some	38·7	44·3
	9	"	27·1	29·2
	10	"	48·2	57·1
	11	"	90·5	107·3
	12	"	56·7	64·9
	15	None	76·1	84·4

*Molecular weight distribution data not available to allow correction to be made.

molecular weight above which molecules of P.V.P. cannot pass through a normal kidney, this threshold must, therefore, be considerably greater than 100,000.

Degradation of P.V.P. in the Body.—When more than about 80% of the infused P.V.P. was recovered a comparison could be made between the molecular weight distribution for the infused P.V.P. and for the sum of all the samples of P.V.P. excreted by any one patient. No allowance has been made for the departure of the recovered P.V.P. from 100%. Fig. 4 shows molecular weight distribution curves for four medical patients and Fig. 5 for two surgical patients. The distribution curves are presented as histograms, since this simplified the calculations. Owing to the very small quantity of P.V.P. of molecular weight above 100,000, the histograms in this region cannot be drawn so that they are distinguishable from the base line.

Wound Dressings.—The presence of P.V.P. in the wound dressing was demonstrated by the iodine method of estimation and the turbidimetric method for the determination of molecular weight distribution, but no quantitative results were obtained owing to the presence of interfering substances.

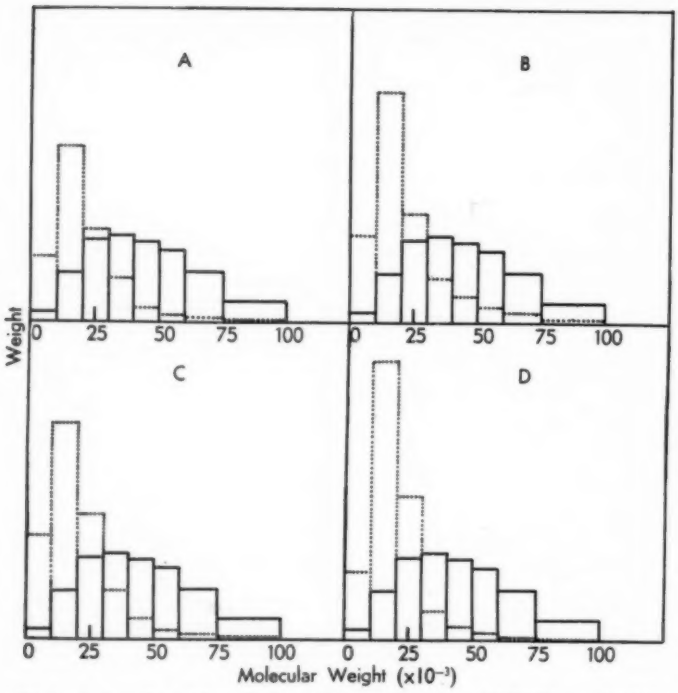

FIG. 4.—A comparison of the molecular weight distribution curves of infused (———) and recovered (.....) P.V.P. for four medical cases. A is patient No. 3, B is patient No. 13, C is patient No. 16, and D is patient No. 18.

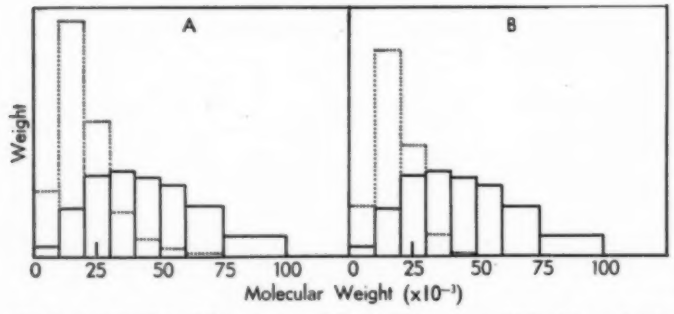

FIG. 5.—A comparison of the molecular weight distribution curves of infused (———) and recovered (.....) P.V.P. for two surgical cases. A is patient No. 11 and B is patient No. 15.

These were also found in dressings from patients not receiving "plasmosan."

Discussion

Although all patients had been selected by the medical staff of the hospitals concerned as free from any kidney disease, some properties of the urine samples, which are listed above in the experimental section, were recorded as supplementary evidence. Apart from the 24-hour volume and creatinine content, there appeared to be no marked departures from normality. Surgical cases differed from medical in that both urine volume and creatinine excretion fluctuated considerably and, on the whole, were appreciably lower than what are regarded as normal values. Such variations, particularly in the immediate post-operative period, might well be due to variations in the patient's fluid intake and disturbance of his nitrogen metabolism. The administration of P.V.P. itself has already been shown by Thrower and Campbell (1951) to exert no ill effect on kidney function.

Low recoveries of P.V.P. in some of the patients studied may have been due to one or more of the following: (1) Loss of urine due to (*a*) incontinence of urine or its contamination by faeces, or (*b*) failure to preserve the urine samples or their loss during examination. (2) Loss of blood into wound dressings and due to menstruation. Such losses were of most significance when they occurred in the first 48 hours after infusion when the concentrations of P.V.P. in blood and urine were greatest. The actual quantity of P.V.P. lost cannot be determined. In all the medical cases the constancy of the 24-hour volume and creatinine content of the urine indicated that losses were negligible. In the surgical cases, however, no such simple interpretation was possible, since the effect of surgery on the urine volume and creatinine excretion was not known. In all surgical cases loss of P.V.P. due to bleeding occurred. For reasons discussed above, such losses could not be assessed and, hence, the

amount of P.V.P. recovered must be regarded as minimal.

In all the cases studied, no differences between the experimental results for male and female patients have been found. It is impossible to say whether the experimental differences between surgical and medical cases, such as the total P.V.P. recovery (Table V), are due to any other causes than the losses of P.V.P. known to occur in the surgical cases.

Apart from its dependence upon blood concentration, the rate at which P.V.P. passes through the kidney must depend inversely upon its molecular weight in order to explain the general shape of the curves in Fig. 2 which give the increase in peak molecular weight with time after infusion. A more detailed analysis of the kinetics of excretion is impossible at present owing to the complicating factor of degradation of P.V.P. Although P.V.P. passes through the kidney very slowly at high molecular weights, evidence has been obtained that P.V.P. of all the molecular weights present in the polymer infused can pass through the kidney. If a threshold molecular weight exists, above which P.V.P. molecules will not pass through the kidney, this is in excess of 100,000. The value of this threshold molecular weight cannot be stated at present with greater accuracy, since the actual weight of P.V.P. of molecular weight greater than 100,000 in "plasmosan" is too small to allow precise measurements to be made. An accurate determination would require excretion studies using P.V.P. of a much higher molecular weight. The excretion of P.V.P. of all the molecular weights present in "plasmosan" is confirmed by the complete recovery of P.V.P. in the urine of those patients in whom loss of P.V.P. may be regarded as negligible (Table V). This complete recovery indicates that disposal of P.V.P. in the body other than by renal excretion is insignificant and that it is not permanently stored in the body. Histological evidence that the grade of P.V.P. used in "plasmosan" is not permanently stored has been found by Whitelaw (see Thrower and Campbell, 1951) in rabbits, and by Haler and co-workers (1953) in a human subject. These experiments refer to plasma concentrations of P.V.P. not grossly in excess of those likely to be found in normal clinical practice. Conflicting evidence concerning storage of other grades of P.V.P. in various animals has been reported by Korth and Heinlein (1943), by Bargmann (1947), by Schoen (1949), by Fresen (1949a), and by Steele, Van Slyke, and Plazin (1952). This lack of agreement between different workers may partly be due to the use of different grades of P.V.P., the molecular weight ranges of which have never been specified, since Fresen (1949b) has shown that the possibility of storage will certainly increase with increasing molecular weight. Other relevant factors may be the weight of P.V.P. per kilogram of body weight infused, and the possibility of differences in the renal excretion between different species of animals.

The threshold molecular weight of excretion for proteins as found by Bayliss, Kerridge, and Russell (1933) is about 68,000 and is much smaller than the value for P.V.P. The explanation of this may be, in part, due to differences in the shape and electric charge of the molecules. Another difference does, however, exist between the plasma proteins and P.V.P. molecules in solution, which may be of importance. No protein molecule can uncoil, and hence change its cross-sectional area, without undergoing irreversible denaturation. Scholtan (1951) has shown that the P.V.P. molecule, like most flexible, long-chain, synthetic polymer molecules, is, in solution, randomly kinked and, unlike the protein molecule, continually undergoes reversible changes in the actual degree of kinking. There is a most probable configuration and cross-sectional area, which, for P.V.P. of a higher molecular weight, may prevent passage through the kidney. Reversible changes in the degree of kinking, however, could yield molecules of smaller cross-sectional area which might then be capable of passing through the kidney. It may be this mechanism which allows P.V.P. molecules of high molecular weight to pass slowly through the kidney.

A comparison of the histograms of Figs. 4 and 5 shows that there is more P.V.P. of a low and less of a high molecular weight recovered in the urine than was infused into the patient. Some form of degradation of P.V.P. molecules must, therefore, occur during their passage through the body, but the mechanism of this change is at present unknown. These observations, which can be due only to a limited degradation of P.V.P., are not in disagreement with those of Steele and his colleagues (1952), who found that after infusions of C^{14} tagged P.V.P. only a very small amount of radioactive carbon dioxide was expired.

Evidence is now available that P.V.P. is very rapidly excreted in the 48 hours after an infusion. Table IV indicates that there is no delay in the beginning of excretion. Table III shows that where there is no loss of P.V.P. between 65 and 90% (corrected weights) is excreted in the first 24 hours after infusion. In the first 12 hours over 50% is excreted. From the above figures it follows that, 24 hours after an infusion, between 10 and

35% of the P.V.P. is left in the body. This must represent the maximum present in the blood stream at 24 hours, since some of the P.V.P. may be, at least temporarily, in other body fluids or tissues.

In conclusion, these excretion studies indicate that the P.V.P. in "plasmosan" as at present constituted is not permanently stored in the body. It would appear that clinically beneficial results can be obtained with a grade of P.V.P. which is excreted as rapidly as the present studies indicate. Whether an improved plasma substitute would result from using a grade of P.V.P. which is excreted more slowly can be decided only by further studies.

Summary

Clinical studies are described of the renal excretion of polyvinylpyrrolidone (P.V.P.) from 15 patients.

Over 90% of the infused P.V.P. can be accounted for in the urine of those patients in whom there was no loss of P.V.P. due to bleeding or to incomplete collection of urine.

If a threshold molecular weight exists above which P.V.P. would not be excreted by the normal kidney, this is in excess of 100,000.

There is a very rapid excretion of P.V.P. in the first 24 hours after an infusion.

There is evidence for degradation of P.V.P. during its passage through the body.

We are indebted to the following without whose cooperation in providing us with the necessary specimens these studies would have been impossible: Mr. G. P. Arden, Heatherwood Hospital, Ascot; Dr. F. Marsh, St. Margaret's Hospital, Epping; Dr. A. Piney, St. Mary's Hospital, Plaistow; Dr. H. W. Salmon, East Ham Memorial Hospital; Professor C. Wilson, The London Hospital. We have been assisted in the measurements in our laboratories by Mr. R. V. Crouch, Mr. D. Gilbert, Mr. S. W. Head, and Mr. T. Moore, and in computing results by the calculating section of the Accounts Division of this firm.

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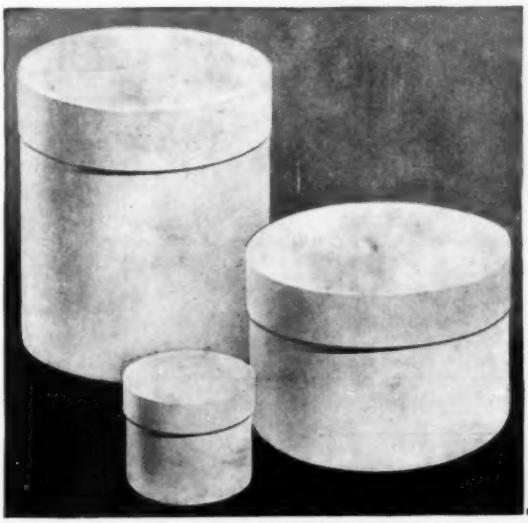

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J. clin. Path. (1954), 7, 261.

THE DETERMINATION OF INULIN BY LITTLE'S METHOD

BY

F. W. DIGGINS AND P. H. SANDERSON

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(RECEIVED FOR PUBLICATION OCTOBER 9, 1953)

When inulin is determined in plasma by methods employing diphenylamine it is found that inulin-free plasma gives rise to an appreciable colour in the blank. Most of this is due to glucose, and the blank can be considerably reduced by treating the plasma with yeast (Alving, Rubin, and Miller, 1939; Harrison, 1942), or by curtailing the time of heating with the diphenylamine reagent, which reduces the colour due to glucose more than that due to inulin (Barnett, Blacket, Depoorter, Sanderson, and Wilson, 1950). Recently Little (1949) has proposed a method in which glucose is destroyed by heating the plasma filtrate in a boiling water-bath at a strongly alkaline reaction.

In the course of some inulin clearance determinations in this laboratory, using Little's method for inulin, unexpectedly low clearances were found in healthy medical students. The source of the error was found to be the heating with alkali, which was causing destruction not only of glucose but also of some of the inulin.

Methods

The inulin used in the clearance experiments was Kerfoot's sterile, pyrogen-free solution, prepared in ampoules for intravenous use. The ampoules were immersed in boiling water just long enough to allow complete re-solution of the precipitated inulin. In preparing standards, a specimen of dry inulin obtained from Messrs. Hopkins and Williams (not A. R.) was used; it was dissolved in water with as little heating as possible. Inulin was determined in plasma filtrates, urine dilutions, and aqueous solutions by Little's method and by Harrison's method. In the first, 4 ml. of solution and 1 ml. 4 N.NaOH. are heated in a boiling water-bath for 10 minutes, tne mixture is cooled, 10 ml. of diphenylamine reagent is added and the mixture heated in the water-bath for a further 45 minutes. In the second method 5 ml. of solution, previously treated with yeast and with deproteinizing agents in the case of plasma, is heated for 45 minutes with 10 ml. diphenylamine reagent in a boiling water-bath. The extinction at 625 mμ was measured in a Unicam spectrophotometer, model S.P. 500. Proteins were removed from plasma by the cadmium hydroxide method (Fujita and Iwatake, 1931) using a dilution of 1 in 20. The diphenylamine reagent used was that described by Harrison (1942).

Results

Table I shows the results obtained by the two methods on one specimen of plasma and one specimen of urine obtained during a clearance determination on a normal healthy subject.

TABLE I

RESULTS OBTAINED BY TWO METHODS

	Inulin (mg./100 ml.)	
	Harrison's Method	Little's Method
Plasma	34·3	25·4
Urine	207	124

It will be noted that, while the figures obtained by Little's method are the lower in both cases, the reduction is far greater in the case of urine. The rate of urine flow at this time was 18·3 ml. per minute, giving an inulin clearance of 89 ml. per minute by Little's method and of 110 by Harrison's method. In three other clearance experiments on young healthy male subjects in whom inulin was estimated by Little's method only, figures of 69 (mean of nine periods), 96 (mean of 10 periods) and 74 (mean of 11 periods) were obtained. In all these experiments, as well as that recorded in Table I, urine dilutions as well as plasma filtrates were treated with alkali before heating with diphenylamine.

The effect of omitting the heating with alkali during the determination of standard inulin solutions was therefore tried. Solutions containing 2.5 mg./100 ml. were prepared (*a*) from the dry inulin sample by weighing, and (*b*) from the solution in ampoules by diluting in accordance with the concentration (10%) stated by the makers. To 4 ml. of each solution 1 ml. of 4 N.NaOH was added and the tubes heated for 10 minutes, cooled,

and treated with diphenylamine as before. A second series of tubes was treated with alkali in this same way, but the heating at this stage was omitted; the diphenylamine reagent was added at once and the colour was developed in the usual way. The results are shown in Table II.

TABLE II
EFFECT OF OMITTING HEATING WITH ALKALI

Inulin Solution	$E^{1\text{ cm.}}_{625}$	
	Heated with Alkali before Diphenylamine	No Heating with Alkali
2·5 mg./100 ml. from dry specimen	0·560, 0·560	0·830, 0·830
2·5 mg./100 ml. from solution in ampoule	0·258, 0·258	0·825, 0·835

The apparent destruction of inulin by alkali was thus 33% in the case of the dry specimen and 69% in the case of the ampoule solution. It was at fir t thought that hydrolysis to fructose might have occurred during the heating required to dissolve the inulin. Table III, however, shows that both specimens of inulin contained only negligible quantities of fermentable carbohydrate, as judged by the diminution in colour produced by previous exposure to a yeast suspension. The ability of the yeast to destroy fructose is shown by the experiment with fructose as substrate.

TABLE III
RESULTS USING FRUCTOSE OR SUBSTRATE

Solution (Final Concentration = 1·25 mg.% in All)	$E^{1\text{ cm.}}_{625}$	
	Treated with Yeast	No Yeast
Inulin from ampoule*	0·445, 0·443	0·435, 0·434
Dry inulin	0·402, 0·402	0·405, 0·399
Fructose	0·054, 0·049	0·420, 0·423

* Diluted on basis of maker's estimate of 10% in original solution.

It was found that the amount of inulin destroyed in a given preparation was not significantly increased by prolonging the period of heating with alkali beyond 10 minutes. Table IV shows the extinctions

TABLE IV
EXTINCTIONS AFTER HEATING DRY SAMPLES WITH ALKALI

Time of Heating with Alkali (Minutes)	$E^{1\text{ cm.}}_{625}$
10	0·592, 0·596
20	0·598, 0·599
30	0·595, 0·580

obtained after heating a solution of the dry sample of inulin with alkali for 10, 20, and 30 minutes.

If a constant proportion of inulin were destroyed in standards, plasma filtrates, and urine dilutions it would be of no moment in the determination of inulin clearance, since what is required is the value of the ratio $\frac{U}{P}$, rather than the absolute value of either numerator or denominator. It was found, however, that the destruction of inulin in plasma filtrates was always less than in urine dilutions and standards (see Table I). The reason for this was not investigated, but it may be significant that when the 4 N.NaOH is added to $Cd(OH)_2$ plasma filtrates a precipitate is formed. (The NaOH used in precipitating proteins was 1 N, as recommended by Little, rather than 1.1 N as originally proposed by Fujita and Iwatake (1931); this minimizes the risk of obtaining an alkaline filtrate, which Shock (1944) has shown to cause serious losses of inulin.) This precipitate is probably $Cd(OH)_2$: if so, it must remove some of the hydroxyl ions from solution, and this would be expected to diminish the rate of destruction of carbohydrate by atmospheric oxygen.

Discussion

It is clear that the preparations of inulin studied, though substantially free from fermentable carbohydrate, were far from homogeneous, since they contained varying amounts of alkali-labile polymers. The data in Little's paper indicate that not more than about 4% of the inulin he used was alkali-labile, but this sample had been specially purified. Dr. J. M. Ledingham has informed us that he has encountered the same difficulty with a dry sample of Kerfoot's inulin in the course of tissue inulin determinations by the method of Ross and Mokotoff (1951), in which treatment with alkali is necessary; up to 50% of the inulin was lost at this stage. By boiling with alkali and recrystallizing he has been able to obtain a product of which not more than 2% is alkali-labile.

The inulin prepared by Messrs. Kerfoot in ampoules for intravenous use appears satisfactory for inulin clearance determinations, since normal values are obtained in normal subjects when the inulin determinations are carried out by the method of Barnett and his colleagues or by Harrison's method. When tissue inulin determinations are required, and heating with alkali cannot be avoided, purification of the inulin on the lines suggested by Dr. Ledingham will be necessary. The experience of the authors suggests that ordinary recrystallization, even if repeated several times, will not overcome the difficulty.

Summary

When inulin which has not been specially purified is estimated by Little's method serious losses may occur during the heating with alkali. The proportion of inulin destroyed may be different in plasma filtrates and urine dilutions, in which case erroneous inulin clearances will be obtained.

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J. clin. Path. (1954), 7, 264.

TECHNICAL METHOD

A FAST GREEN METHOD FOR STAINING RED CELLS

BY

G. P. XUEREB* AND R. BEESLEY

From the Nuffield Department of Surgery, University of Oxford, and the Department of Pathology, Radcliffe Infirmary, Oxford

(RECEIVED FOR PUBLICATION JULY 31, 1953)

In a study of the vascular pattern of the human pituitary body carried out by one of us (Xuereb, 1953) the benzidine methods (Lepehne, 1919; Pickworth, 1934) of staining red cells were chiefly used. Satisfactory results were obtained, but the use of these methods is accompanied by two well-known disadvantages: the stained preparations gradually fade and the technique cannot be applied to old, formalin-fixed material. In order to overcome these difficulties the method of fuchsin staining (Eros, 1941) was also used, but it did not yield satisfactory results when applied to the pituitary body as a whole. We therefore substituted various other dyes for the acid fuchsin, and good results were obtained by the use of fast green. The following staining procedure was evolved using a dilute aqueous solution of this dye.

The pituitary bodies were fixed in 10% formol saline for a period varying from two to 10 weeks, and the staining method was applied to frozen sections ranging in thickness from 100 to 120 μ. The sections are washed in distilled water and then immersed in an aqueous solution of fast green (5 drops of a stock 2% solution of fast green to 50 ml. distilled water) for 30 to 45 minutes. The sections are then differentiated in a phenol-lithium carbonate solution (5 drops of 0.5% lithium carbonate solution to 50 ml. of 1% phenol). This stage is controlled under the microscope and the differentiation allowed to proceed until the network of blood vessels becomes apparent (15–30 minutes). After washing in distilled water the sections are dehydrated in absolute alcohol, cleared in a terpineol-xylol mixture (3 parts terpineol to 2 parts xylol) and mounted in dammar. In the finished preparations the red blood cells appear dark green against a colourless background.

The pituitary preparations have now retained the intensity of the stain for two years. Equally satisfactory results were obtained from tissues preserved in formol saline for as long as 12 months. These advantages outweigh the fact that the fast green method is somewhat time consuming owing to microscopic control of differentiation. It is, however, more rapid than the acid fuchsin method.

FIG. 1.—Fast green preparation showing the vascular pattern in the various components of the pituitary body (female, aged 76 years). Sagittal section through neural lobe (top), posterior part of pars distalis (bottom) and infundibular stem (centre). Note relatively sparse, delicate vessels in the circular area of chromophobe adenoma (lower bottom) contrasting with the surrounding dense network of sinusoids (×12).

*Working with a grant from the Nuffield Foundation.

When the latter technique was applied to the pituitary body it was found that by careful differentiation either the vessels within its epithelial or those within its neural portions could be shown clearly. With the fast green method, however, it was possible to make preparations in which the vessels of both adeno- and neuro-hypophysis stand out equally well.

Lendrum's (1949) kiton red method was given an extensive trial on thin and thick paraffin and frozen sections of the human pituitary body, and the following conclusions were drawn: (1) Fast green is better than kiton red for staining the blood vessels in thick frozen sections (120 μ) and thick paraffin sections (20 μ). The fast green adheres more tenaciously to the red blood cells and consequently allows better differentiation from the densely cellular background. (2) Fast green is as good a method as kiton red when applied to thin paraffin (7 μ) and thin frozen (12 μ) sections. (3) Differentiation is quicker with fast green.

The fast green method of staining red cells has proved useful in determining the arrangement of the blood vessels in all the components of the hypophysis cerebri, not only under normal conditions but also in areas affected by disease processes (Fig. 1). This method has also given us good results with renal tissues, and is now being adapted for the study of the cerebral capillary bed.

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J. clin. Path. (1954), **7**, 266.

REVIEWS

Lectures on General Pathology. Edited by Sir Howard Florey. (Pp. 733.) London: Lloyd-Luke Ltd. 1954.

The Oxford medical student who has read the Honour School of Physiology receives a course in general pathology and bacteriology before proceeding to his clinical studies. The course lasts for two terms of eight weeks each, and some idea of its character is gleaned by this collection of 37 lectures given by ten teachers. Since the design is clearly that of bridging the gap between these two phases of the student's training, the lectures should be read from this angle. It is pertinent to ask whether they are given in the order published. If so, it appears strange that the early (and excellent) lectures on acute inflammation are widely separated from those on healing and chronic inflammation, which come at the end. In many chapters, too, the subject-matter is handled in a manner more suited to the advanced student: it may be that, for the purpose of publication, much detail has been added that the authors would not impose verbally on the beginner. Indeed many of the lectures contain more meat than might be considered digestible at one session, even by the most acquisitive student

The preface emphasizes that the whole field of general pathology is not surveyed. It is true, for example, that no lectures will be found on neoplastic disease: an odd omission, in view of its importance. Since a very proper bias is to be found in general towards advanced physiology and the experimental approach, would it not be appropriate to give the student some outline of experimental work on carcinogenesis? The answer may be that, in restricting the field, it is preferable to deal in considerable detail with the fundamental aspects of inflammation, immunity, and kindred topics. There can be no quarrel with this in principle. But, in detail, is it not better for the beginner to be versed in such important circulatory disorders as embolism and infarction than to be laden with details concerning the biological effects of irradiation? There may be a good answer to this.

It must be added that the general level of treatment of the topics selected is both excellent and up to date. The Oxford student who is sufficiently receptive to digest the contents of this book has indeed been given a flying start to his clinical work. The volume is admirably produced and beautifully illustrated.

DOROTHY S. RUSSELL.

Biological Effects of External X and Gamma Radiation. Part I. Edited by Raymond E. Zirkle. (Pp. xxvi + 530.) Illustrated. 58*s*. London: McGraw-Hill. 1954.

A great deal of experimental work was done in the United States during the war years with the aim of defining safe conditions for exposure to irradiation and of elucidating the pathogenesis and possible treatment of radiation damage. The volume under review contains the substance of a number of reports on the effects of penetrating radiation, which, even when no longer subject to security regulations, have not been easily accessible. The inevitable pressure during the war for a quick answer, and the dispersal of the authors when the war was over and often before the work was written up, is reflected in some unevenness of quality, as the editor points out. The attempts to find a biochemical basis for irradiation damage were largely negative. More valuable perhaps are the chapters on the haematological responses of rabbits to single exposures, and especially those on the effects of long-continued irradiation of mice, guinea-pigs, and rabbits for the whole of their life span. Sterilization because of damage to testis or ovary, cytopenia following bone marrow damage, and carcinogenesis were observed in all, but there were marked quantitative differences between species in the amount of radiation required to produce a similar amount of damage. The discussion of how to apply these findings to the problem of human protection leaves transparently clear the difficulties of transferring chronic toxicological data from animal experiments to man when the biological mechanisms are so imperfectly understood.

R. H. MOLE.

J. clin. Path. (1954), **7**, 267.

RISKS FROM CHRONIC IRRADIATION AND THEIR HAEMATOLOGICAL CONTROL

BY

R. H. MOLE

From the Medical Research Council's Radiobiological Research Unit, Atomic Energy Research Establishment, Harwell

(RECEIVED FOR PUBLICATION MAY 17, 1954)

Although it is now standard practice to do periodic blood counts on people regularly exposed to radiation in the course of their work no criteria exist by which the amount of radiation damage or its severity can be assessed unambiguously from the blood picture. This sometimes makes the interpretation of the counts a difficult and anxious matter. My purpose here is to consider in a general way whether these blood counts can in fact ever serve the purpose for which they are intended, and to raise the question whether it is appropriate for the clinical pathologist to do them.

In the field of atomic energy a serious effort has been made to see that workers run no risk. It is impossible to prevent all exposure to irradiation, and safe working levels are usually expressed in terms of maximum permissible dose (m.p.d.) rates; their calculation is described by Neary (1952). The m.p.d. is the rate at which radiation may be received every working day throughout a lifetime without causing harm. This does not imply a complete absence of damage. The gardener's hands lose tactile sensitivity and may even develop a characteristic deformity from potting plants (Cohen,1947), but this amount of damage is accepted as not harmful. The m.p.d. takes into account not only the possibility of harm to the individual but also the possibility of harm to the race by reason of the genetic changes produced by radiation (Catcheside, 1950). The clinical pathologist, however, is concerned primarily with the individual, and this paper is concerned with the value of blood counts to the individual occupationally exposed to radiation.

When the "Recommendations for Radiation Protection" were agreed upon internationally in 1931 changes in the blood count were regarded as more than just damage; they were considered as evidence of harm done to the individual (Recommendations of the International X-ray and Radium Protection Commission, 1932). By definition therefore exposure to the maximum permissible dose rate of radiation should not produce blood changes, and the value of blood counts would then be to detect over-exposure. Now exposure to radiation can be quantitatively measured by means of specially treated photographic films carried by the individual worker (film badges) or by radiation meters, so that blood counts should show changes only when working conditions allow of over-exposure, and be of value only when discipline fails and the worker does not carry his film. The clinical pathologist is then being called in to detect engineering and disciplinary failures. If we accept that this is an appropriate role for him to play we ought to make sure that his tests are sensitive enough to do the job.

Changes in the Blood Count due to Radiation

Fig. 1 shows schematically what patterns of change might be observed in some constituent of the blood during chronic exposure to irradiation. If the ability to repair were not damaged too much one might expect, as in (*a*), an initial drop, due to the destructive effect of radiation, without any

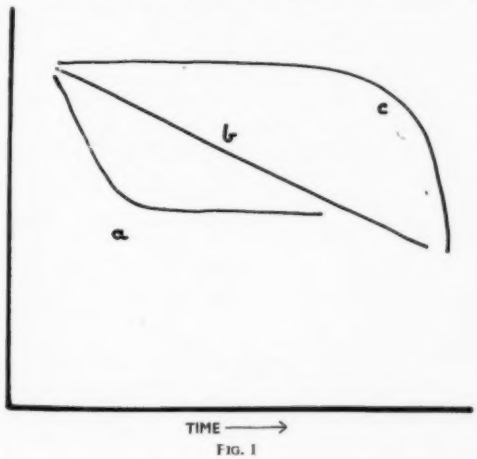

FIG. 1

further decrease subsequently, since the rate of repair would then be able to keep up with the rate of destruction. If the ability to repair were damaged sufficiently one would expect a steadily progressive fall as in (*b*). And if repair could keep up with destruction until the ability to repair was exhausted, then a curve like (*c*) might be found. Clearly periodic checks on the blood could only be of use in detecting damage if the changes due to irradiation followed patterns (*a*) or (*b*) and it seems to have been tacitly assumed that there would be a progressive decrease as in (*b*) with progressing damage.

Experimental

In actual fact the changes observed seem to follow one or other of the other two patterns (*a*) and (*c*). For example, when mice were irradiated continuously the blood lymphocyte count dropped in the first few months and then remained steady at a fairly constant fraction of the control value (Lorenz, Eschenbrenner, Heston, and Uphoff, 1951). The neutrophils were not similarly decreased. No changes in the blood count, however, were detectable at levels of irradiation of 50 times the m.p.d.* (Lorenz, Heston, Jacobson, Eschenbrenner, Shimkin, Deringer, and Doniger, 1946). The dog is more sensitive to radiation, and Fig. 2 shows the changes

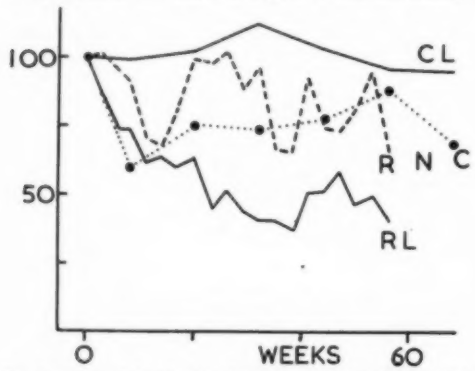

FIG. 2.—Effect of chronic *x*-irradiation on the white cell count of the dog (3 r daily for one year). C = control, R = irradiated, N = neutrophil (heterophil), L = lymphocyte. Each count is given as a percentage of the initial control value. (Redrawn from data of Ingram and Mason, 1950.)

produced by 60 times the m.p.d. (Ingram and Mason, 1950). The lymphocyte count again followed pattern (*a*) of Fig. 1, and again this was a more sensitive indicator of radiation effects than the heterophil count, where there was no difference from the control group. Fig. 3 shows the changes in the guinea-pig, the most radio-sensitive animal haematologically speaking, irradiated at 80 and 20 times the m.p.d. (Lorenz *et al.*, 1946). Neutrophil and lymphocyte counts were decreased to 50% below the controls at the higher dose rate, and to about 20% below at the lower dose rate, though this smaller change is only evident statistically. But white

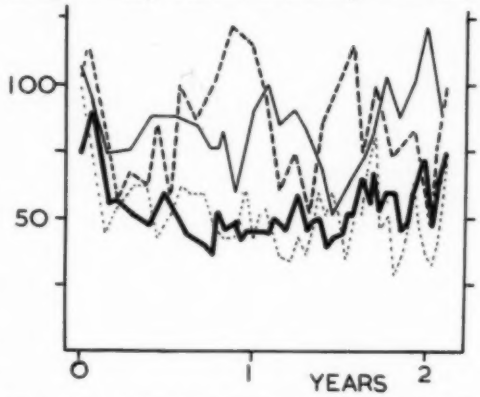

FIG. 3.—Effect of chronic irradiation on the white cell count of the guinea-pig. Continuous lines lymphocytes, broken lines heterophils. The two upper curves are from animals irradiated at 1.1 r daily, the two lower curves are from animals irradiated at 4.4 r daily. The counts are given as percentages of the counts of unirradiated controls examined about the same time. (Redrawn from data of Lorenz and others, 1946.)

counts would have provided a poor control of irradiation hazards for these guinea-pigs. The mean drop of 20% below the control value is just detectable from inspection (Fig. 3), yet at the end of two years deaths among guinea-pigs exposed at this dose rate were double the controls and at the higher dose rate 75% of the whole group was dead. There was no progressive decrease in cell count to indicate increasing damage.

When applying these experimental findings to the clinical problem three things have to be borne in mind. The observed changes at 20 to 80 times the m.p.d. (Figs. 2 and 3) were clearly visible because groups of animals were used to establish the points: the changes would be much harder or impossible to detect with certainty in a single individual, granted the inevitable and natural fluctuations between repeated counts. Next, weekly dose rates of 20 times the m.p.d. and more just should not occur where people work. And lastly in a variety of animals a lymphopenia has been at least as sensitive a sign of damage as a neutropenia, whereas the orthodox teaching is that the earliest quantitative change in men exposed to irradiation is a decrease in neutrophils without any change in the number of lymphocytes.

* In order to avoid introducing technical terms unnecessarily, all doses in this paper are given in multiples of the human maximum permissible dose rate (0.05 r daily).

Before considering the human evidence on which this view was based, the more serious haematological effects of irradiation will be illustrated. When a monkey is given radioactive strontium it localizes in the bones and irradiates the bone marrow continuously (Edington, Judd, and Ward, 1953). In such an animal the white count was unaffected even on the day of death (Fig. 4). There was a

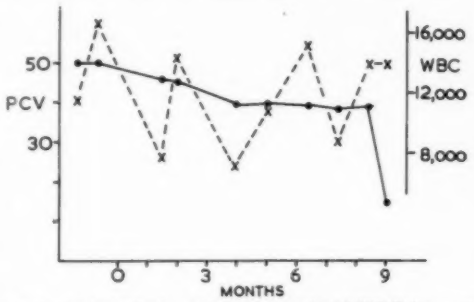

FIG. 4.—Packed cell volume (●——●) and total W.B.C. (× – – ×) in a monkey given $_{90}$Sr (Judd and Ward, 1954).

very slow fall in the haemoglobin and red count for nine months and then a quite suddenly developing and fatal anaemia. The pattern of change is of the third type (*c*) of Fig. 1, and it is clear that in this case the white count gave no prior warning that the marrow was aplastic.

Clinical

The same seems to be true of the human cases of aplastic anaemia caused by radiation; in those too the white count gave no prior warning of what was to come. By the time the anaemia had become severe the white count was usually low (though not always; 4,700 leucocytes per c.mm. on the day of death in the case described by Émile-Weil and Lacassagne, 1925) as in aplastic anaemia from whatever cause, but anaemia and leukopenia developed quickly. In only a few of the cases of anaemia due to external radiation described in the literature (listed by Lambin, 1930) is information available about the blood picture shortly before the onset of the illness; the white count was then within normal limits (Table I and Fig. 5). Now it is historically true that it was these deaths from aplastic anaemia which initiated in this country the first agreed "Recommendations for Radiation Protection," later adopted internationally, and which made blood counts seem a most important protective measure. It seems clear, however, that it is unlikely that these tragedies would have been prevented by blood counts, though they would most certainly have been prevented had any kind of physical monitoring of working conditions been available in those early days.

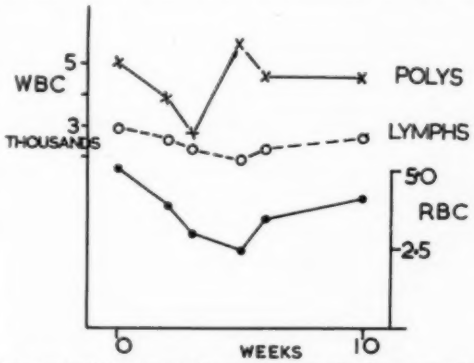

FIG. 5.—Serial blood counts in a non-fatal case of radiation anaemia. (Data from Carman and Miller, 1924.)

TABLE I

BLOOD PICTURE SHORTLY BEFORE ONSET OF FATAL APLASTIC ANAEMIA IN THREE RADIATION WORKERS

	Case of Larkins (1921)		Case of Faber (1923)		Case A of Mottram (1920)*	
Months before death	5	1½	10	1	9	2½
Haemoglobin (%) ..	85	54		56	95	45
R.B.C. (m/c.mm.) ..	4·2	2·7	4·6	3·8		2·2
W.B.C. (thousands/c.mm.)						
Total	7,200	1,600	5,000	4,200	3,900	1,200
Neutrophils ..	4,300	300	Normal	2,400	2,000	590
Lymphocytes ..	2,700	1,200	„	1,500	1,700	640

* I am indebted to Mr. Burgh and Mount Vernon Hospital for the hitherto unpublished data of Mottram's case A. No early blood counts are available on his other two cases. It is noteworthy that the last illness in all three cases described by Mottram (1920) was of short duration. This alone is not positive evidence that human illness follows pattern (*c*) of Fig. 1, but for Case A this was clearly true. She is stated to have been in excellent health during the whole of her eight years' occupational exposure and her illness began at the end of a two months' holiday.

Mottram (1921) found a real neutropenia amongst hospital staff working in admittedly bad conditions of exposure, which improved when exposure to radiation was reduced. But the two individuals with really low neutrophil counts (500 and 1,000 per c.mm.) had low lymphocyte counts too (900 and 700 per c.mm.). In the three examples of the effects of work with radium which Mottram (1931) gave the fall in lymphocytes was proportionally greater than in neutrophils. At the time when he found these changes working conditions were bad enough to kill (Mottram, 1920), so that, although the radiation doses to which Mottram's subjects were exposed are unknown, they will have been many times the present-day m.p.d., i.e., as high as those experienced by animals showing similar changes.

What was the evidence then that led to the orthodox teaching already mentioned, that neutropenia was the commonest early sign of over-exposure to radiation? The findings of the earlier workers will not be criticized, but this interpretation of their findings will be. In the last 20 years the standards of evidence in medicine have become considerably more stringent. Further, two important facts have only recently been fully appreciated: the considerable variability to be expected in doing serial white counts on an individual, and second the curious and still unexplained fact that, when a series of blood counts is made on an individual, there is often a progressive change in the white count so that the real baseline normal is not reached until after the first one or more counts have been done (Chamberlain, Turner, and Williams, 1952).

When it was first being realized that radiation might affect the blood count, any change whatsoever in someone exposed to radiation was naturally attributed to the radiations, so that monocytosis, eosinophilia, leukopenia, leucocytosis, anaemia, and polycythaemia, to mention only some of the findings, were all considered as evidence of radiation damage. This attitude may be illustrated in the work of Amundsen (1924), who found a "relative lymphocytosis" in a group of people working with radiation in a hospital. He expressed surprise that he found the same change in the hospital maids, the night nurse, and two medical students who had been working in the hospital for only a month, and concluded that this only showed how sensitive blood changes were to exposure to radiation. The alternative conclusion, that since the "abnormality" was found in those exposed to scarcely any radiation it did not signify radiation damage at all, was apparently not considered.

There was also too great a reliance on differential percentages. Lavedan (1929), for instance, described as showing "no change" progressive falls in total white count of 29 to 42% (his Cases 35 to 40), whereas a change of less than 10% in the proportion of neutrophils without a change in the total white count was often thought important (his Cases 1 to 7). There were always a few authors, for example, Barclay and Cox (1928), who failed to satisfy themselves that changes in the blood picture did occur in those working with radiation under conditions which were then thought reasonable but which would now probably be thought to give daily exposures at several times the m.p.d.

Ten years later after much propaganda occupational exposure in hospitals was considerably less. Nevertheless it was still believed that leukopenia was being produced. Fig. 6 includes a reproduction of Fig. 3A from Goodfellow (1935) giving the mean white count of people working with radium in Manchester. Twice over there appeared to be a progressive leukopenia when working conditions were bad with a return towards normal when these were improved. Goodfellow investigated 28 subjects altogether, but during the three years of the investigation these subjects were coming and going, so that different individuals contributed to the mean white count at different times. This would seem to make hazardous any deduction from changes in the group mean white count. Only four subjects in fact worked throughout the whole period, and when their mean white count is plotted (Fig. 6) there is no obvious change with worsening or improvement in working conditions. These were sufficiently bad at times for marked skin changes to have occurred in several subjects.

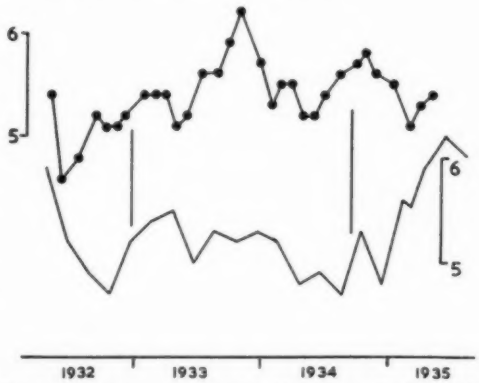

FIG. 6.—The lower curve is the mean white cell count (in thousands) of all persons working with radium (redrawn from Goodfellow, 1935). The upper curve is the mean white cell count of the four individuals who worked with radium throughout the period (data from Goodfellow, 1935). The vertical lines indicate the two occasions when additions were made to the staff and duties were reorganized so as to reduce exposure to radiation. At the beginning of 1933 the hospital was enlarged, and this led to progressively increasing work with radium, nullifying the effects of the first reorganization.

Goodfellow's other evidence for the leukopenic effects of radiation was the fall in the white count during the first few weeks of work with radium. Recently it has been shown (Chamberlain and others, 1952) that just such an initial fall in the neutrophil count is to be expected when serial counts are carried out on healthy individuals not exposed to irradiation at all. The lymphocyte count is not affected in this way. Thus an early fall in the white count and an increase in the percentage of lymphocytes cannot be considered evidence of radiation damage, as it was by Goodfellow and many other authors. It seems as if it is this early physiological

fall in neutrophils which was responsible for the belief that exposure to radiation produced neutropenia. The same phenomenon is sometimes but not always seen in animal experiments (cf. Fig. 2).

One and only one of Goodfellow's subjects is recorded as having worked with excessive doses of radiation. His blood count during an experiment with 0.8 curie of radium is shown in Fig. 7, and it

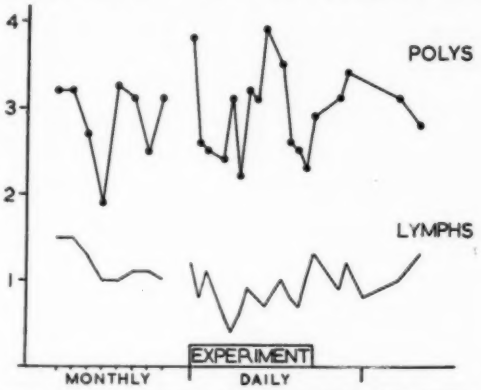

FIG. 7.—White cell counts (in thousands) of an individual doing an experiment with 800 mg. radium (data from Goodfellow, 1935).

is interesting that his lymphocyte count seemed to decrease during exposure while his neutrophil count did not. This might be considered more of an acute than a chronic exposure, however.

Value of Blood Counts to Individuals Exposed to Radiation

The evidence shows that animal experiments and human experience agree. Radiation exposure at levels greater than, say, 20 times the m.p.d. is likely to produce a real but relatively small drop in neutrophils and/or lymphocytes. How can the clinical pathologist use this information to help the individual? The temporal variation in the white count of individuals is such that a 30% fall is to be found at random in 10% of successive counts (Chamberlain and others, 1952). So when the pathologist finds a low count in someone, gives him a holiday, and then finds his count has come up to normal, it is not legitimate to conclude that the subject had too much irradiation and then recovered. We have also seen that serious aplastic anaemia can occur without necessarily being preceded by much of a decrease in white cells. My own opinion is that blood counts are not of any help to the individual who is working in properly controlled conditions, i.e., in circumstances where there is physical control over the radiation exposure.

Such physical control by film badges and/or radiation meters should be the rule in every hospital. Radiation exposure may nevertheless occur in unexpected ways. Fig. 8 shows the distribution of radiation around a patient with 100 mg. of radium in the cervix (Mayneord, 1951). The hands of a nurse making the bed of such a patient may be irradiated by up to 50 m.p.d., so that a few hours' bed-making a week might give her hands the permissible dose. This risk was revealed by a physicist using physical methods.

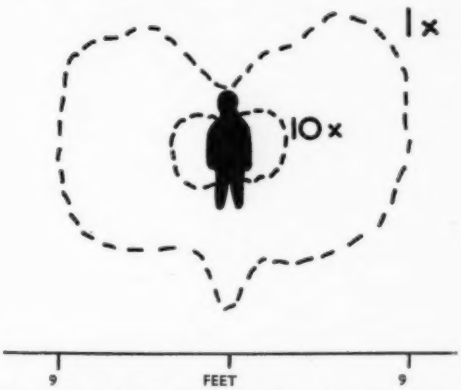

FIG. 8.—Distribution of radiation around a patient with 100 mg. of radium in the cervix. The contours show where 1 and 10 m.p.d. are reached. (Redrawn from Mayneord, 1951.)

Radiation exposure at a place like the Atomic Energy Research Establishment, Harwell, is rigorously controlled. It is in industry in general and especially in universities that people may be very lax about their own protection. For varying reasons they want to get on with their jobs and cannot be bothered—an admirable trait. So Whitby (1949) was able to find definite changes in the blood picture in people working in a university physics laboratory. The weekly dose of his illustrative case was more than 10 times the m.p.d. when the blood changes occurred. In view of our ignorance of the exact relation between radiation dose and change in the blood picture in man it is well worth while to seize every opportunity to investigate individuals in whom exposure is measured physically and is sufficiently frequent or great to lead to blood changes. But it is clear that such exposure is over-exposure and really should not occur. It is perhaps necessary to consider how far the doctor should attempt to protect someone against himself.

Those who are responsible for the clinical care of persons engaged in work with radiation are well

aware that the worker may fail to carry his film badge, carelessly or deliberately, but that he cannot leave his bone marrow behind him when he goes to expose himself unnecessarily. For someone with clinical responsibility it is a great comfort to feel that there is something which labels the individual and could show up a breakdown in routine protection measures, but the evidence suggests that for radiation workers the comfort provided by blood counts is illusory (Chamberlain and others, 1952). Discussion of morphological changes in blood cells has been deliberately avoided, since there is certainly no general agreement at present that any particular morphological change is characteristic of radiation damage, let alone of harm.

Relation of Marrow Damage to Peripheral Blood Count

The general picture of the effects of radiation discussed here has some similarity to the effects of drugs toxic to the bone marrow. Sulphonamides, thiourea and its derivatives, and other drugs can produce a serious or fatal aplastic anaemia with little warning. To prevent its occurrence routine white counts at regular intervals are often recommended, but the value of such counts is often called in question, since when the dangerous changes occur their onset is so sudden.

If we are to consider changes in the peripheral blood as evidence of harm to the individual we must believe that the peripheral changes reflect in some way damage to the bone marrow. But is this really an acceptable general principle? Do we believe that someone whose white count is 6,000 per c.mm. has a more active marrow than someone with a count of 5,000 and that the naturally more active marrow will be more resistant to damage by radiation (or drugs)? When there are undoubted changes in the numbers of cells in the peripheral blood due to radiation exposure how can we tell whether the changes were caused by alteration in marrow activity or by some other effect of radiation, and in either case how do we decide that the observed changes indicate harm rather than an acceptable degree of damage? This is one of the questions that have to be faced when considering whether some individuals, especially those whose counts fall at either end of the normal distribution of the population, are peculiarly susceptible to radiation.* Strong views have often been expressed about this, but it is difficult to see how the question can be answered from a consideration of blood counts alone. The only clear evidence of harm to the marrow is the occurrence of serious marrow disease, and aplastic anaemia from radiation stopped occurring in the early 1920s, since when the m.p.d. has twice been lowered.

This question of how far changes in the peripheral blood reflect damage to the bone marrow or lymph nodes must also be faced when considering the meaning of the reported changes in the average count of a group of people exposed to some fraction or small multiple of the m.p.d. (for summary see Stone, 1952). It is often considered that, even if blood counts are of no value to the individual, the group average of a number of people similarly exposed may reveal over-exposure or radiation damage. Chamberlain and others (1952) found a difference of 124 cells per c.mm. in the mean lymphocyte count of exposed and unexposed workers ($P<0.02$). The measured radiation dose in those exposed averaged less than 5% of the m.p.d. However real in a statistical sense this difference may be, and granted that it is valid to conclude that it was due to radiation and not to some other difference between the two groups, does this difference matter or is it trivial, in the sense in which changes in a gardener's hands may be trivial? Clearly with larger and larger numbers of counts to average smaller and smaller differences in means become statistically demonstrable, but something outside the statistics is needed to tell whether the differences are meaningful or not.

Harmful Effects of Chronic Irradiation

It would not be right to give any impression that radiation is not harmful. It must be treated with great respect, not least because the harm sometimes takes 20 years to show itself. Hundreds of cases of malignant disease* have been recorded as caused by radiation (against some 30 of fatal anaemia), and chronic irradiation at dose levels less than that needed to affect the blood count can have markedly damaging effects on the gonads (Fig. 9). This increased sensitivity of the gonads as compared with the blood count seems to be generally true experimentally (for summary see Stone, 1952) and clinically. In the only two human cases of fatal anaemia in which the testis was examined at necropsy, spermatogenesis was found to be completely absent (Gavazzeni and Minelli, 1914; Wegelin, 1930), and in a third case

* Turner (1953) showed that the "natural" sickness rates of those with persistently low white counts were less than those with normal white counts.

* Leukaemia is also a consequence of occupational exposure to radiation (March, 1950). In one reported case of leukaemia occurring in a radiation worker where serial blood counts were being done (Weitz, 1938) no warning signs of radiation damage were observed, but occupational exposure had been so short that it seems unlikely that the leukaemia was caused by radiation.

the atrophy was recognized in life long before the onset of the fatal anaemia (Faber, 1923). Some modern Archimedes may develop a technique for weighing the testis *in vivo*, but until he does gonadal

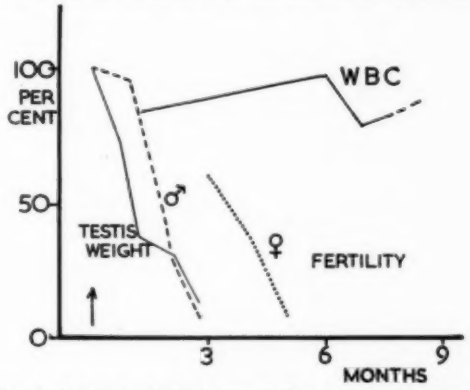

FIG. 9.—Schematic demonstration of effects of chronic neutron irradiation on the white cell count, testis weight, and fertility of male and female mice (Neary, Munson, and Mole, 1954).

damage cannot be easily assessed in man. So that the greater sensitivity of the gonads may be another reason for preferring that radiation hazards should be measured physically rather than controlled by laboratory examination of the exposed individual.

Function of the Clinical Pathologist in Detecting Environmental Hazards

The doing of blood counts of those exposed to irradiation is just one example of the use of the clinical laboratory in the control of environmental hazards in general. During the last hundred years the bacteriological hazards to health have been steadily decreasing because of public measures of control, measures which do not concern the individual directly. But as civilization increases in complexity, physical and chemical hazards multiply, sometimes quite unexpectedly. Some of these hazards are completely controlled by public measures, but others are controlled by subjecting individuals to laboratory examination, what may be called private or personal control of a hazard. We look for stippled red cells in lead workers, at serum cholinesterase levels in people spraying modern insecticides. These hazards are bound to go on multiplying, and with the increase in number and kind of potential hazard there is bound to be an increase in the laboratory examinations which clinical pathologists will be asked to make. The more clinical pathologists are likely to become involved in this kind of work the more important it is to scrutinize beforehand the evidence that their work will do what it is supposed to do.

Is it true to say that if enough money and care were spent on engineering control of a hazard there would be no need for the clinical pathologist to look for evidence of damage in exposed individuals? In the atomic energy field it has been repeatedly said about different processes that it would be impossible to arrange the work so that the workers did not get a larger dose of radiation than they should. When the limitation of the irradiation has been insisted on, however, an engineering solution has always been found, though always at some economic cost. At some point someone has to decide, usually on most inadequate evidence, when damage becomes harm, and how much human life and suffering are worth in cash. Something under a thousand men die each year in this country from the occupational risks of mining coal by accidents and pneumoconiosis (Loutit, 1954). The same degree of risk would entail a death from electrocution or irradiation every three to five years amongst the radiologists, radiographers, and hospital physicists of this country. Clearly this amount of risk is not considered reasonable, and one advantage, let us hope, of the development of atomic energy will be a saving of human life in the getting of industrial and domestic power.

Summary

It is suggested that regular blood counts are of no value to those occupationally exposed to radiation provided there is regular physical measurement of the doses received. The evidence that blood counts are of value has not withstood careful examination. Changes in the peripheral blood by themselves and without further evidence should not be assumed to mirror damage to the bone marrow. Damage to the gonads seems to be a more sensitive index of exposure to radiation.

A clear distinction should be borne in mind between damage and harm; clinical experience is the only guide to the amount of damage which is harmful.

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THE APPEARANCE AND SIGNIFICANCE OF TISSUE MAST CELLS IN HUMAN BONE MARROW

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Despite an increasingly vast literature concerning the examination of bone marrow, scant attention has been paid to the occurrence of tissue mast cells in human marrow or to their significance when present. Only recently have mast cells been described in the marrow of a small number of cases by Rohr (1949), Leitner (1948, 1949), Bremy (1950), Tischendorf and Hartmann (1950), Fadem (1951), Koszewski (1952), and Hayhoe (1953). Generally the patients had a severe marrow disturbance, which was frequently of the hypo- or aplastic variety, and, in the opinion of both Undritz (1946a and b) and Bremy (1950), the appearance of mast cells in the marrow indicates severe marrow depression and is of diagnostic and prognostic significance. However, Williams (1952), using marrow biopsy particle smears, was able to demonstrate mast cells in as many as 56 (17%) of 325 marrows.

Fixed tissue sections of routine marrow aspirates were used in the present work, the object of which was to observe the frequency with which tissue mast cells occur in human marrow and to try to assess their significance.

Methods and Materials

The material for this investigation was taken from consecutive marrow aspirates submitted routinely to this laboratory for examination, the marrow samples being aspirated by standard methods, in most instances from the sternum. While sections of some of the earlier material were prepared according to the method described by Cappell, Hutchison, and Smith (1947), for the majority the modification detailed by Hutchison (1953) was used. The sections were stained with 0.001% aqueous toluidine blue, and while no difference was found to exist between the staining properties of mast cells in sections prepared by either method, the advantage of the latter lies in the fact that the blood, which always contaminates and dilutes aspirates, is removed in the processing and consequently a fragment of pure marrow concentrate is obtained.

Tissue mast cells and basophil leucocytes (blood mast cells) differ morphologically, having in common only the metachromatic staining property of their granules. As the granules of these latter cells have been found to be extremely soluble in water and alcohol they are not seen in fixed tissue sections when an aqueous or alcoholic fixative has been used, and the basophil leucocytes therefore cannot be identified.

Sections have been compared by enumerating the mast cells in microscopic fields at a constant magnification (× 320), and the marrows placed in one or other of the following groups. Those marrows in which no mast cells were seen form Group 1: Group 2 contains marrows with an average of up to five mast cells per microscopic field, while in Group 3 an average of more than five mast cells was seen in each field. Only occasionally was it found difficult to decide into which group a marrow should be placed.

Incidence

Apart from necropsy material, which will be mentioned later, 269 marrow aspirates from 230 patients were examined. No mast cells were seen in 68 (30%) patients (Group 1). Group 2 contained the largest number with 139 (60%) cases, while the remaining 23 (10%) fell into Group 3. The pathological diagnoses of the patients in each group are detailed in Table I.

Twenty-seven patients had more than one marrow aspiration. In 21 the marrows were classified under the same group each time, in one case the proportion of mast cells remaining constant over 10 marrow examinations. In the remaining six patients the number of mast cells varied only slightly.

The advantage of sections over smears in the demonstration of tissue mast cells was striking. No quantitative comparison was made, but examining smears for their presence was time consuming and generally unrewarding. Even when shown to be abundant in the sections they were usually absent or scanty in the smears.

Morphology

The morphological characteristics of mast cells seen in marrow have been described elsewhere

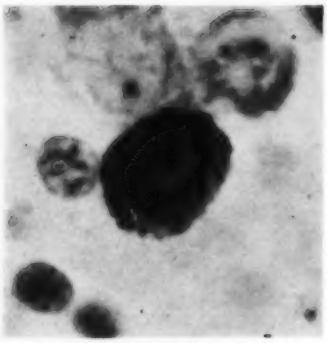


FIG. 1a

FIG. 1b

FIG. 1.—Details of two mast cells, one (*b*) showing an elongated cytoplasmic process × 1,900 (toluidine blue).

(Breeny, 1950; Fadem, 1951). While the majority of the cells are rounded or ovoid (Fig. 1) bizarre forms are frequently seen. Some show pseudopodia-like extensions, many are elongated and spindle-shaped, while others have a blunted head containing the nucleus with the remainder of the cytoplasm drawn out into a long filamentous process, occasionally seen to be bifid. Cytoplasmic granulations frequently overlaid the nucleus and often completely obscured it. Mitotic division and binucleated forms were not seen.

Distribution

Mast cells are known to occur throughout the body and particularly in relation to the adventitia of small blood vessels. This has certainly been observed in the marrow, but for the most part they are by no means confined to this situation. They occur somewhat irregularly; in the reticular framework of the marrow, among the haemopoietic elements, and even stretched over the surface of fat cells. The irregularity of distribution is occasionally pronounced and a small area with abundant mast cells may be observed in a marrow which otherwise contains but few mast cells.

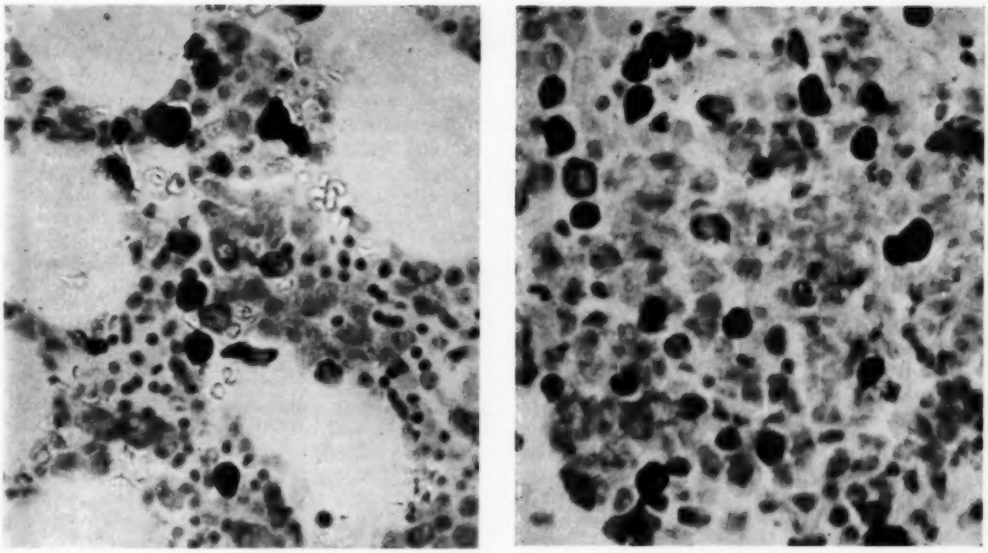
In sections of the whole thickness of bone obtained at necropsy the mast cells are often seen to be most numerous in and about the endosteum but scanty towards the centre of the medullary cavity. This was also noted by Ellis (1949) at the necropsy of a 12-month-old infant with urticaria pigmentosa.

Age

Very few children were included in this material, the majority of the patients being in the fifth to seventh decades, the ages ranging from 11 to 81 years.

TABLE I
TYPES OF CASES COMPRISING EACH GROUP

Type of Case	Group 1: No Mast Cells	Group 2: Up to 5 Mast Cells per Field	Group 3: More than 5 Mast Cells per Field
Pernicious anaemia ..	20	38	6
Iron-deficiency anaemia ..	12	43	5
Haemolytic anaemia ..	—	3	—
Reticulosis	4	6	1
Carcinoma	3	10	2
Chronic infection	2	5	—
Myelomatosis	3	2	—
Leukaemia	8	1	1
Miscellaneous	16 Polycythaemia, myxoedema (2 each); aregenerative anaemia, cerebro-vascular degeneration, red cell aplasia, osteoporosis, scurvy, anxiety state, nephritis, mitral stenosis, histoplasmosis, malabsorption syndrome, gastro-enteritis, myeloid metaplasia (1 each)	31 Vascular disease (4), cirrhosis, Banti syndrome, B.T. malaria, nephritis, treated breast carcinoma (2 each); aplastic anaemia, essential thrombocytopenic purpura, normal, sprue, pneumonia, splenic neutropenia, sarcoidosis, ulcerative colitis, steatorrhoea, peripheral neuritis, disseminated sclerosis, infectious mononucleosis, haemachromatosis, rheumatoid arthritis, renal cyst, peptic ulcer, cardiac failure (1 each)	8 Aregenerative anaemia, unexplained continued pyrexia, uraemia, Banti syndrome, aplastic anaemia, osteoporosis, oesophageal stricture, cachexia (1 each)
Total ..	68	139	23



(*a*) (*b*)

FIG. 2.—(*a*) Large numbers of tissue mast cells in a marrow of normal erythropoiesis × 750 (toluidine blue). (*b*) Many tissue mast cells in a hyperplastic marrow (pernicious anaemia) × 650 (toluidine blue).

Marrow was obtained at necropsy from various sites from 31 infants and children dying from a wide variety of pathological conditions. Mast cells were seen in only six cases and were always scanty.

Iron

Iron is stored in stainable form in the reticulo-endothelial cells of the marrow in varying amounts dependent on the existing pathological conditions. Marrow sections from 185 of the aspirates had been stained for iron (Hutchison, 1953), but no correlation could be found between the iron content of the marrow and the number of mast cells seen.

Haemopoiesis

At an early stage in this investigation it seemed that the numbers of mast cells in the marrow might depend on a purely mechanical factor and be regulated simply by the degree of marrow cellularity, i.e., extreme marrow hyperplasia might crowd out mast cells from the marrow and hypoplasia allow their free multiplication. That this is not the case is readily seen in Table II, where it is obvious that there is no association between the numbers of mast cells and the extent of haemopoiesis (Fig. 2).

TABLE II

NUMBER OF CASES IN EACH GROUP COMPARED WITH THE DEGREE OF HAEMOPOIESIS

Group	Degree of Haemopoiesis			
	Hypocellular	Normal	Moderate Hyperplasia	Marked Hyperplasia
One ..	1	18	18	31
Two ..	3	59	44	33
Three ..	2	7	6	8

Lymph Follicles

In sections from 21 (9%) of the cases lymphoid follicles were observed in the marrow. These follicles are minute foci or aggregates of small lymphocytes with no germinal centre. Mast cells appear constantly in the periphery of these follicles, but are never observed in the centre (Fig. 3). The number of mast cells varied considerably; occasionally they were scanty, generally plentiful, and sometimes abundant. While constantly present in these lymphoid follicles, transitional forms between mast cells and lymphocytes were not observed, and the lymphocyte and plasma cell origin of mast cells expounded by Downey (1913) cannot be refuted or confirmed.

Discussion

It is our belief that tissue mast cells form a normal constituent of human bone marrow. By making use of fixed tissue sections, mast cells have

been observed in as many as 70% of the marrows examined. The pathological state of the patients from which these marrows were aspirated represent a wide variety of conditions, but some of the marrows themselves show no qualitative abnormality. Further, mast cells have been demonstrated in marrow obtained at necropsy from a few normal healthy individuals who have died from injuries sustained in various forms of accidents.

Mast cells were not observed in 30% of the marrows, but it is probable that when scanty their presence or absence in any given marrow section may well be governed by the selection inevitable in a small sample.

In a small proportion of the present cases the numbers of observed mast cells must be considered abnormally high in relation to the numbers seen in the great majority of the marrows. The cause of this increase is not immediately apparent although several suggestions have been put forward. Mast cell granules are generally believed to contain heparin (Jorpes, Holmgren, and Wilander, 1937; Jorpes, 1946; Oliver, Bloom and Mangieri, 1947; Köksal, 1953), and Paff, Bloom, and Reilly (1947) thought that the inhibitory effect of heparin on growth (Goerner, 1931) explained the failure of all cells but mast cells to grow when tissue from dog mastocytoma was cultured, but Macdougall and Riley (1954) attribute this simply to the great preponderance of mast cells present in the original tissue. Fadem (1951) considered it possible that a similar relationship might exist between mast cell accumulation and hypoplasia of the marrow in some cases, the marrow growth being inhibited by the excessive amount of heparin elaborated by the increased numbers of mast cells in the marrow. However, if this be the case, the marrow depression is but secondary to the increase of mast cells and the explanation of this increase remains unsolved.

In the present series marrow hypoplasia was observed in only two of the aspirates showing mast cell accumulation, erythropoiesis in the remainder being either normal or hyperplastic. Further, in addition to the six cases of severe marrow depression included in Table I, material from marrow depressive states from eight necropsies and two bone marrow trephines was examined, mast cell accumulation being observed in only five of these 16 cases. From this it is clear that hypoplasia of the marrow *per se* is neither a prerequisite for mast cell accumulation nor an inevitable consequence of it, although the presence of mast cells is frequently associated with some form of marrow depression in the cases recorded in the literature (Bremy, 1950; Leitner, 1949; Rohr, 1949; Fadem, 1951; Hayhoe, 1953).

Describing "myelitis chronica interstitialis" Rohr (1948) suggested that in the early stages of the more serious form there is an irreversible proliferation of the marrow stromal fibrocytes and reticulum cells together with plasma cells and mast cells, sometimes leading to a resistant secondary marrow depression, and later to the final extreme picture of myelofibrosis. The marrow stromal change was thought to be the local manifestation of a systemic reaction, possibly of an allergic nature, to some infection or noxious agent. Certainly mast cells are known to accumulate in other sites in relation to areas of chronic inflammation, and it is interesting to note that recent work affords strong presumptive evidence that the granules of mast cells contain histamine in addition to heparin (Riley and West, 1952; Riley, 1953). Thus their association with anaphylactic-allergic states would not be unexpected.

Brief details of all the present cases showing mast cell accumulation are given in the table in the appendix. While this table contains a few cases of marrow depressive states similar to those described by Rohr (1948, 1949) and Bremy (1950), on the whole it affords little evidence to support their view that the appearance of

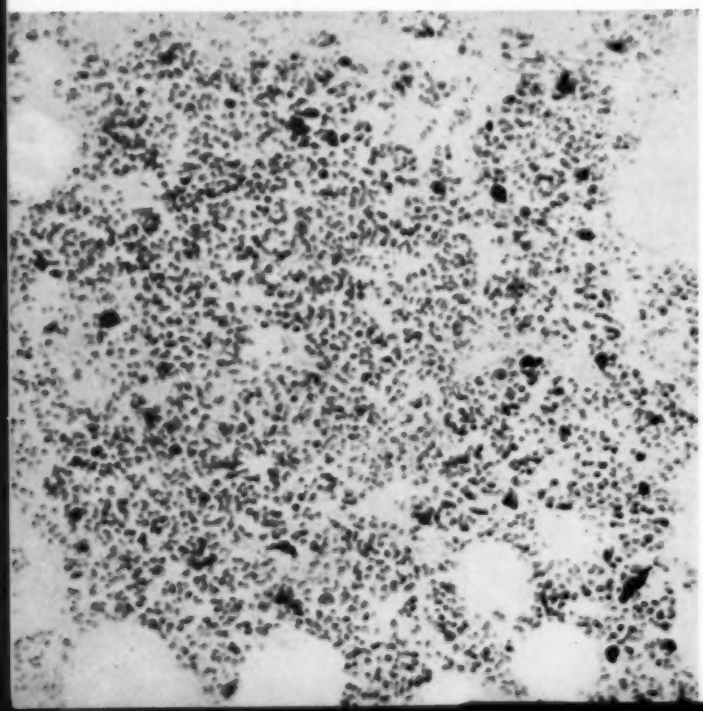


FIG. 3.—Lymphoid follicle in marrow with many tissue mast cells towards the periphery × 225 (toluidine blue).

mast cells in the marrow is part of a reaction to an allergic process. There is little in the cases which might suggest any infective agent, either past or present, and the majority of the marrows are hyperplastic with no increase in the amount of stromal elements or plasma cells.

Anaemia of moderate to severe degree is the only factor common to all the cases although many do also show some degree of splenomegaly. But neither anaemia nor splenomegaly, alone or in combination, are essential for, or invariably accompanied by, mast cell proliferation.

Adrenal cortical hypofunction or adrenalectomy is said to result in marrow hypoplasia (Gordon and Charipper, 1947; Feldman, Rachmilewitz, Stein, and Stein, 1953), and the number of mast cells in the skin, muscle, and heart of intact rats was observed by Cavallero and Braccini (1951) to be markedly reduced following the injection of cortisone, but the effect of adrenalectomy on these cells is not known. It seems therefore theoretically possible that adrenal cortical hypofunction may be at least partly responsible for the increase of mast cells in the marrow of some cases exhibiting marrow depression, but the response to A.C.T.H. and cortisone in such patients is as yet most disappointing (Wintrobe, 1951; Spaet, Rosenthal, and Dameshek, 1951; Davidson, Girdwood, and Swan, 1952; Medical Research Council Report, 1953), and no change was observed in the number of mast cells in the marrow of two cases receiving treatment with A.C.T.H. (Williams, 1952).

Summary and Conclusions

Two hundred and sixty-nine marrow aspirates from 230 patients have been specifically examined for mast cells and they have been classified in three grades according to the numbers present. The advantage of section over smears for this purpose is stressed.

No mast cells were seen in 68 (30%) of the patients, a few were present in 139 (60%), and in 23 (10%) they were abundant.

The presence and numbers of mast cells in the marrow bore no relation to the iron content of the marrow or to the degree of erythropoiesis.

Lymph follicles were seen in 21 (9%) of the marrows and mast cells were then constantly observed in their periphery; they were, however, not confined to this situation. The morphology and distribution of the mast cells have been described.

Mast cells are believed to form one of the normal constituents of human bone marrow and they have been observed to accumulate abnormally in a small proportion of cases. The cause of this increase is not clear and no single factor can as yet be held responsible. While these cases show a varying degree of anaemia associated generally with a moderate to severe marrow disturbance, the earlier explanations offered and the gloomy prognosis previously associated with the appearance of mast cells in the marrow cannot be substantiated.

I should like to thank the physicians and surgeons in charge of wards at the Western Infirmary for the clinical details, Mr. M. Fitch, F.I.M.L.T., for technical assistance, and Mr. G. Kerr for the photomicrographs.

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APPENDIX

BRIEF DETAILS OF ALL CASES SHOWING MAST CELL PROLIFERATION

Case No.	Age	Sex	Hb (g. %)	R.B.C. (m./c.mm.)	Erythropoiesis	Diagnosis	Remarks
1	50	M	10·5	3·35	Hyperplasia +	Bronchial carcinoma; skeletal metastases; specific aortitis	Wassermann reaction positive
2	72	M	5·7	1·60	,, +++	Pernicious anaemia	Megaloblastic marrow
3	48	F	8·3	1·95	,, +++	,, ,,	,, ,,
4	69	F	3·0	0·99	,, ++	,, ,,	,, ,,
5	50	M	4·0	1·05	Severe hypoplasia	Aplastic ,,	Death in 4/12 from first symptom: no cause identified: few cells, other than lymphocytes, in marrow
6	62	F	9·7	3·12	Normal	Atypical rheumatoid arthritis	Mild pyrexia; joint pains; W.B.C. 8,000; laboratory tests negative; responded eventually to Butazolidin
7	56	F	6·0	1·40	Hyperplasia +++	Pernicious anaemia	Megaloblastic marrow
8	56	F	7·0	1·41	,, +++	,, ,,	,, ,,
9	46	F	6·0	3·55	,, ++	Iron-deficiency anaemia	—
10	66	M	6·0	1·98	Normal	Carcinomatosis	Carcinomatosis; hepatic secondaries proven a laparatomy? Bronchial primary. No necropsy
11	53	M	10·0	3·90	,,	Cachexia	6/12 weakness and weight loss: E.S.R. 108/121; hepatomegaly; achlorhydria; skeleton normal; plasma proteins normal; G.I. tract normal; pyrexia responded to antibiotics
12	53	M	5·9	1·03	Hyperplasia +++	Pernicious anaemia	Megaloblastic marrow
13	44	F	6·1	1·72	,, +++	Aregenerative anaemia	W.B.C. 2,900/c.mm.; marrow-megakaryocytes ++; scanty erythroid series; maturation failure of both erythroid and myeloid series; no stromal or plasma cell increase. Required blood transfusions every 6/52
14	65	F	9·0	2·4	,, ++	Oesophageal stricture	Simple oesophageal ulceration. W.R. neg.
15	56	F	—	—	Normal	Osteoporosis	Epileptic. Long-standing generalized osteoporosis with multiple fractures and collapse of several lumbar vertebrae
16	50	M	9·7	3·3	Hyperplasia ++	Aleukaemic lymphatic leukaemia	Confirmed at necropsy
17	52	F	8·0	4·2	,, ++	Banti's syndrome	Iron deficient; W.B.C. 2,600; splenomegaly; thymol turbidity 6 units; plasma proteins normal. Several hundred mast cells/field
18	46	F	5·6	3·2	,, +	Iron-deficiency anaemia	—
19	62	M	4·5	2·9	,, ++	,, ,, ,,	—
20	81	M	9·0	3·0	Hypoplasia +	Uraemia	Hypertensive. Prostatic obstruction; blood urea 174 mg. %. Plasma proteins normal
21	26	F	5·3	3·1	Hyperplasia +++	Iron-deficiency anaemia	—
22	64	F	10·3	4·35	Normal	P.U.O.	Unexplained pyrexia for 4 months. Albumin 3·1; globulin 3·4 g./100 ml. serum. E.S.R. 120 mm. in 1 hour. W.B.C. 11,000/c.mm. Normal differential. All other clinical and laboratory findings normal
23	62	F	10·5	5·1	Normal	Iron-deficiency anaemia	—
Marrow trephine material							
24	71	M	5·7	1·9	Hyperplasia +++	Reticulo-sarcoma	Bone trephine suggested myelofibrosis with many reticulum and connective tissue cells. Necropsy, reticulo-sarcoma with extensive marrow spread
Necropsy material							
25	44	M	—	—	Hypoplasia	Myelofibrosis	Aplastic anaemia requiring transfusions for 11 years; hepatomegaly and splenomegaly; at necropsy, myelofibrosis and osteosclerosis
26	67	F	—	—	,,	Agranulocytosis	Drug agranulocytosis: W.B.C. 50/c.mm. Ulceration of fauces and rectum: death from pulmonary embolism
27	56	M	—	—	Normal	Aregenerative anaemia	No details available

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ON THE APPEARANCE IN THE MARROW OF IRON ADMINISTERED INTRAVENOUSLY

BY

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Intravenous administration of saccharated oxide of iron is now frequently used in the treatment of hypochromic anaemia where either iron given by mouth is resisted (Hawkins, Peeney, and Cooke, 1950) or rapid improvement is especially important, as in pregnancy (Govan and Scott, 1949), or before surgical operation (Briscoe, 1952). It is therefore surprising that no observations have yet been published regarding the subsequent distribution of the iron in the human body in such cases. It is the purpose of this paper to give a brief description of some observations we have made on the appearance of histologically demonstrable iron in the sternal marrow at different intervals after the intravenous administration of varying amounts of saccharated oxide of iron to patients with iron-deficiency anaemia.

Material and Methods

On occasion over the past few years the opportunity has presented itself of examining the marrow of patients undergoing intravenous iron therapy.

It would serve no useful purpose to give details of the haematological and general medical findings in these patients; instead the initial haemoglobin level and a brief clinical diagnosis are listed in each case (Tables I and II). In all cases the response to iron subsequently confirmed the diagnosis of iron deficiency.

When the decision to give intravenous iron was made sternal puncture was carried out in the usual way and the marrow fragments concentrated, sectioned, and stained for iron (Cappell, Hutchison, and Smith, 1947; Hutchison, 1953). In accordance with the findings of Davidson and Jennison (1952) and Hutchison (1953) in iron-deficiency anaemia, no stainable iron was detected in these initial aspirates. This not only provided immediate confirmation of the diagnosis but prevented the administration of iron when it was not required and so eliminated the possibility of therapeutic siderosis, a material consideration when using parenteral iron (Dameshek, 1950; Holly, 1951).

The injections were begun as soon as the initial marrow sample had been examined. The iron preparation used was "ferrivenin" and the initial dose for the patients listed in Table I was 50 mg. followed by 100 mg., usually on successive days. In the first two cases the second marrow sample was not secured until 24 hours after administration of the total dose necessary to correct the anaemia. In the remaining 11 patients shown in Table I the second marrow sample was secured earlier, when progressively smaller amounts had been given of the total dose of iron judged necessary to meet the patient's haemoglobin deficit, but

TABLE I
CLINICAL FINDINGS

Clinical Findings	Hb (g.%)	Total I.V. Iron (mg.)	Interval between Start Therapy and Second Marrow Examination	Marrow Iron
Idiopathic. M.C.H.C. 22%. M. 34 years	7·1	1,150	49 days	+
Idiopathic. M.C.H.C. 28%. F. 23 years	7·0	1,000	20 ,,	+
Cancer caecum. M.C.H.C. 22%. FOB++. M. 61 years	6·2	450	5 ,,	+
Idiopathic. M.C.H.C. 27%. F. 41 years	8·2	450*	3 ,,	+
Idiopathic. M.C.H.C. 27%. Cerebral neoplasm ..	8·2	450*	3 ,,	+
Bleeding peptic ulcer ..	6·5	350	4 ,,	+
Idiopathic iron-deficiency anaemia	7·2	350	4 ,,	+
Idiopathic. M.C.H.C. 24%. F. 51 years	5·1	250	3 ,,	+
Idiopathic. M.C.H.C. 26%. F. 47 years	7·2	150	3 ,,	+
Idiopathic. M.C.H.C. 24%. F. 46 years	5·9	150	3 ,,	+
5 pregnancies in 5 years. C.I. 0.67. F. 30 years	4·9	150	3 ,,	+
Idiopathic. M.C.H.C. 22%. F. 31 years	4·8	150	2 ,,	+
Cancer caecum. M.C.H.C. 26%. FOB++. M. 73 years	6·75	150	30 hours	Neg.

* These patients each received two injections each of 200 mg.

TABLE II
CLINICAL FINDINGS IN SECOND GROUP OF CASES

Clinical Findings	Hb (g. %)	Interval (hours) between Start Therapy and Second Marrow Examination	Marrow Iron
Melaena, cause unknown. M.C.H.C. 21%. F. 34 years	5·15	48	+
Bleeding peptic ulcer. M. 56 years	9·1	36	+
Idiopathic. M.C.H.C. 24%. F. 26 years	5·9	24	Neg.
Idiopathic. M.C.H.C. 25%. M. 60 years	6·86	24	+Trace
Idiopathic hypochromic anaemia koilonychia. F. 47 years	8·75	24	+
Menorrhagia. M.C.H.C. 23%. F. 34 years ..	6·2	24	+
Idiopathic. Peripheral neuritis. F. 33 years	10·5	5	Neg.

always, unless otherwise stated, 24 hours after the last therapeutic injection.

When it was found that stainable iron could usually be demonstrated in the marrow following as small an amount as 150 mg. a second group was studied. These patients were given a single injection of 100 mg. and the second marrow sample was collected at varying intervals thereafter as is shown in Table II together with the results of examination for iron. The slight irregularity in the findings is probably due to the technical difficulty of demonstrating small amounts of iron in minute tissue samples.

Comment

The appearance of stainable iron in previously negative marrows after administration of only a mere fraction of the total dose necessary to correct the anaemia is a significant observation. Saccharated oxide of iron forms colloidal aggregates and has been shown by Cappell (1930) in the mouse to be phagocytosed by the reticulo-endothelial system after intravenous injection.

In the human subject we have found that the iron is deposited in the cells lining the vascular sinusoids (Fig. 1), the distribution that one would expect from a similar, i.e., non-specific, phagocytic mechanism. Furthermore it appears as discrete granules in cells the remainder of whose cytoplasm is negative to the Prussian blue reaction. This particulate form is also suggestive of ingestion and storage of a colloidal preparation presented to the cells in a very low concentration in the blood.

The short interval of 24 hours which elapsed between administration of the 100-mg. dose and the appearance of stainable iron in the marrow is also in keeping with phagocytic activity, for, 24 hours following the injection, the serum iron had returned substantially to the pre-injection level in these patients indicating that the blood had been cleared of the iron put into it.

The particular importance of these findings is that they confirm the supposition based on animal experiments that saccharated oxide of iron given intravenously rapidly becomes deposited in the reticulo-endothelial system. It is clear from the therapeutic responses which the patients subsequently showed that the iron was afterwards utilized. It is not known in what form precisely iron is taken up by developing erythroblasts for incorporation in the haemoglobin molecule, but there is evidence from radio-iron studies both *in vivo* (Huff, Elmlinger, Garcia, Oda, Cockrell, and Lawrence, 1951) and in tissue culture work that the iron from simple ferric salts conjugated with globulin can very quickly be synthesized into haemoglobin. The rapidity of the therapeutic response to intravenous iron in cases of proved iron deficiency suggests that the transfer from the reticulo-endothelial system to the developing cells takes place extremely rapidly. Our observations suggest that this may possibly be accounted for at least in part by the initial distribution of the iron, some of it having been introduced directly into the sites where it is to be utilized.

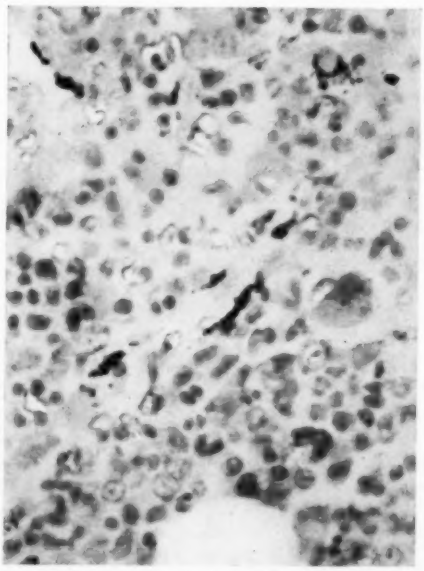

FIG. 1.—Iron granules in sinus-lining cells four days after 350 mg. iron intravenously. Prussian blue × 680.

Summary

In 20 patients the marrow was examined at different intervals following the administration of varying amounts of iron intravenously for hypochromic anaemia. The appearance and distribution of the stainable iron found are thought to be accounted for by the phagocytic activities of the reticulo-endothelial system.

We would like to thank Dr. J. A. W. McCluskie, physician in charge of wards, for his continued interest in this investigation and Mr. M. Fitch for his careful work in the preparation of the histological sections of the sternal aspirates.

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THE EFFECT OF THERAPEUTIC RADIO-PHOSPHORUS ON THE AFFINITY OF HAEMOGLOBIN FOR OXYGEN IN PATIENTS WITH POLYCYTHAEMIA VERA

BY

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Detailed studies in polycythaemia vera (Berk, Burchinal, Wood, and Castle, 1948 ; Wasserman, Dobson, and Lawrence, 1949 ; Schwartz and Stats, 1949) have failed to show any abnormality which might support the theory that the disorder is due to anoxaemia, and there is a large measure of agreement that the condition results from a primary hyperplasia of the haemopoietic tissue of the body. Treatment has therefore been mainly directed at reducing this excessive activity, and radio-phosphorus has now become widely accepted (Lawrence, Berlin, and Huff, 1953) as a highly effective therapeutic agent. While there are numerous morphological studies (Lawrence *et al.*, 1953) on the effect of radio-phosphorus upon the bone marrow and formed elements of the blood, there are no observations, as far as we are aware, on the effect of this form of radiation on the functional capacity of the red cell. We report here alterations in the affinity of haemoglobin for oxygen in patients with polycythaemia vera after the administration of radio-phosphorus.

Material and Methods

Six patients with polycythaemia vera were studied. The details of the cases are given in the accompanying table. In the two cases most intensively investigated (Cases 1 and 2) the circulating blood mass was reduced by repeated venesections before the administration of radio-phosphorus (P^{32}). In these cases the preliminary

TABLE I

EFFECT OF RADIO-PHOSPHORUS ON THE OXYGEN DISSOCIATION IN POLYCYTHAEMIA VERA

Case No.	Patient		Time of Estimation in Relation to P^{32}	Dosage of P^{32} (mc.)	R.B.C. (m. per c.mm.)	Packed Cell Volume (c.cm. per 100 ml.)	HbO_2 Capacity (Vol.%)	% HbO_2 at Various Partial Pressures* of O_2 (mm. Hg) and Constant pCO_2 (40 mm. Hg)				Total CO_2s† (Vol. %)	Plasma pH	Comments
	Sex	Age						pO_2 100	pO_2 60	pO_2 40	pO_2 20			
1	M	60	Immed. before	—	6·5	58	22	97	89	73	32	57	7·4	
			10 days after	6	6·78	60	26·5	96	89	75	33	58	7·41	6 pints of blood removed by venesection before P^{32} and before initial oxygen dissociation curve
			3 weeks ,,		6·95	59	24	90	85	70	37	58	7·41	
			7 weeks ,,		7·0	60	25	91	84	69	36	—	—	
			3 months ,,		7·65	63	27·5	97	90	73	32	56	7·39	
			4 months ,,		7·24	61	26·5	97	—	74	32	58	7·41	
2	M	66	1 month before		8·43	66	30	99	—	75	33	59	7·42	
			Immed. ,,		6·65	56	24	98	90	73	33	—	—	5 pints of blood removed by venesection before P^{32}
			2 weeks after	6	6·55	—	22·5	97	—	73	32	—	—	
			1 month ,,		6·58	—	22	88	85	68	36	55	7·38	
			2 months ,,		4·44	39	15	96	—	77	33	—	—	
3	F	51	Before		7·10	72	—	—	—	—	—	—	—	4 pints of blood removed by venesection before P^{32}
			2 months after	6	5·27	46	21	92	—	70	37	58	7·41	
			4 ,, ,,		4·0	—	15	98	—	77	34	—	—	
4	F	56	Before		7·83	—	—	—	—	—	—	—	—	1 pint of blood removed by venesection before P^{32}
			14 months after	6	5·3	52	20·5	97	88	74	33	56	7·39	
5	M	40	Before		8·47	77	—	—	—	—	—	—	—	6 pints of blood removed by venesection before P^{32}
			7 months after	6	5·7	50	21	96	89	74	33	58	7·41	
6	F	59	Immed. before		8·27	66	26·5	97	—	72	31	55	7·38	
			2 weeks after	3	8·4	65	27	97	—	—	30	—	—	
			1 month ,,		7·3	67	24·5	97	—	74	32	57	7·4	No venesections
			7 weeks ,,		8·3	65	25·5	97	—	72	30	—	—	

* The partial pressure of oxygen and carbon dioxide are expressed to the nearest whole figure.
† Total CO_2s = the total carbon dioxide of true plasma from oxygenated blood at a CO_2 partial pressure of 40 mm. Hg.

gas studies were performed after the venesections but before the administration of radio-phosphorus. Radio-phosphorus was given intravenously as Na_2HPO_4 in a dosage of 3 to 6 millicuries. The time relationship of the blood gas studies to the administration of the radio-phosphorus is indicated in the table.

The techniques used for the blood gas studies were as previously described (Valtis and Kennedy, 1954).

Results

The detailed results are given in Table I and Fig. 1.

All the oxygen dissociation curves performed before radio-phosphorus was given were normal (Cases 1, 2, and 6). Normal oxygen dissociation

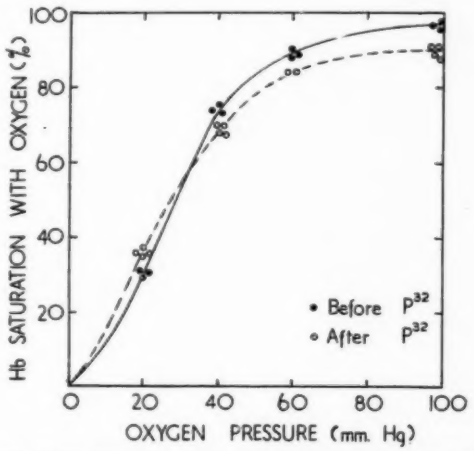

FIG. 1.—Position of the oxygen dissociation curve in three cases of polycythaemia vera before and one to two months after P^{32}.

curves were also obtained in the two patients (Cases 4 and 5) to whom a dose of radio-phosphorus had been given several months before the present study.

In contrast to these findings, the oxygen dissociation curves were abnormal in Cases 1 and 2 one to two months after the administration of 6 millicuries of radio-phosphorus. A similarly abnormal curve was also obtained two months after the administration of 6 millicuries of radio-phosphorus in Case 3; unfortunately, in this case, a curve was not performed before radio-phosphorus was given. The curves obtained in these three cases, during the one to two months after radio-phosphorus therapy, crossed the normal curve at approximately 30 mm. Hg partial pressure of oxygen, the upper part of the curve being lower than normal and the lower part higher than normal. Oxygen dissociation curves performed in these patients more than two months after radio-phosphorus therapy were normal.

In Case 6, only a small dose, 3 millicuries, of radio-phosphorus was given; no alteration in the oxygen dissociation curve was observed during the subsequent two months.

It will be seen from the table that a satisfactory therapeutic depression of the red cell level followed radio-phosphorus therapy in Cases 2, 3, 4, and 5.

Discussion

Our results show that in three cases of polycythaemia vera which received 6 millicuries of radio-phosphorus the oxygen dissociation curve was temporarily altered, the change being evident in the period between one and two months after radio-phosphorus. No alteration was noted during this period in the patient who received 3 millicuries of radio-phosphorus or in the two cases in which blood gas studies were performed for the first time several months after radio-phosphorus.

The alteration of the oxygen dissociation curve observed was not the parallel alteration in position, which we have observed on numerous occasions (Valtis and Kennedy, 1954), resulting from changes in *p*H or electrolytes (Barcroft, 1928), from alterations in the water content of the red cell (Henderson, 1928), or from anaemia (Richards and Strauss, 1927). The fundamental work of Barcroft suggests that changes in the shape of the curve, of the type that we have observed, indicate that there is some alteration in the basic elements of haemoglobin. The fact that the alteration of the oxygen dissociation curve is not present until some weeks after the administration of radio-phosphorus suggests that it may be related in some way to internal radiation from radio-phosphorus on the red cell precursors in the bone marrow. The therapeutic activity of radio-phosphorus, in the form Na_2HPO_4, appears to be due to its synthesis into important cell constituents, including ribo- and thymo-nucleic acids of multiplying cells (Mitchell, 1951), and the importance of these substances in the metabolism of factors necessary for red cell maturation is also well known (Vilter, Horrigan, Mueller, Jarrold, Vilter, Hawkins, and Seaman, 1950). It is conceivable that as a result of this action there is some alteration in the synthesis of haemoglobin in the red cells produced under the influence of internal radiation which could be responsible for the abnormality we have observed.

It is interesting to speculate whether the effect on the oxygen dissociation curve is peculiar to the radiation produced by radio-phosphorus, or whether it would also be produced by *x*-ray radiation, particularly of the skeleton. As far as we are aware, there are no studies which have been published in this field.

It is unlikely that the sulphur products resulting from the decay of radio-phosphorus are the cause of the alterations in the oxygen dissociation curve since the quantities involved are very small and, moreover, 50% of the administered radio-phosphorus is excreted or decayed within 14 days: after six weeks only about 10% of the substance remains (Hevesy, 1948).

As a result of the alteration in the oxygen dissociation curve which we have demonstrated, the blood of the patient will, for a period, take up oxygen less readily in the lungs and release oxygen less readily to the tissues. The magnitude of the changes is not sufficient, however, to affect significantly the oxygenation of the tissues, particularly if, as in the patients we have studied, the haemoglobin level is normal or even above normal.

Summary

The oxygen transport by the blood in polycythaemia vera has been studied before and after the administration of radio-phosphorus. A temporary alteration, apparent between one and two months after radio-phosphorus, in the oxygen dissociation curve was observed; the affinity of haemoglobin for oxygen at high oxygen partial pressures (greater than 60 mm. Hg) was decreased and the affinity at low oxygen partial pressures (less than 40 mm. Hg) was increased. These changes indicate a reduced saturation of haemoglobin with oxygen in the lungs and a reduced release of oxygen to the tissues.

The causation of the changes is briefly discussed.

We wish to express our thanks to Professor L. J. Davis for his advice and encouragement throughout this study; the radio-phosphorus was administered by Dr. E. M. McGirr. One of us (D.J.V.) was enabled to undertake this work by scholarships from the E.C.A. for Greece and from the University of Thessalonica. Much of the cost of apparatus and materials was provided by the Rankin Fund of the University of Glasgow.

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THE THROMBOPLASTIC ACTIVITY OF RUSSELL'S VIPER VENOM AND ITS RELATIONSHIP TO FACTOR VII

BY

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The thromboplastic activity of Russell's viper venom was first recognized to be of practical value when it was used by Macfarlane and Barnett (1934) as a powerful local haemostatic agent in the treatment of haemophilia.

Fullerton (1940) substituted Russell's viper venom for rabbit-brain thromboplastin in his modification of Quick's one-stage prothrombin time, since viper venom could be obtained in a more convenient and more stable form than brain extract It soon became apparent, however, that the two methods gave widely different results. Wilson (1947) and many other workers found that in cases treated with dicoumarol much higher plasma prothrombin values were obtained with venom than with brain thromboplastin, and, in fact, Fullerton's modification could be dangerous, since it gave inadequate warning of prothrombin values low enough to cause haemorrhage.

It has been shown by Macfarlane, Trevan, and Attwood (1941) that a lipoid substance in the plasma is necessary for the action of viper venom, and an excess of lipoid, such as occurs after a fatty meal, shortens the prothrombin time with venom but not with brain thromboplastin (Fullerton and Anastasopoulos, 1949).

A further source of the difference in action of the two thromboplastins became apparent when it was recognized that the one-stage prothrombin time measured not only prothrombin but also the two accelerator factors which have been termed Factor V (Owren, 1947) and Factor VII (Koller, Loeliger, and Duckert, 1951).

It seemed possible that venom and brain extract reacted to these factors in a different manner, and the relationship of Factor VII to these thromboplastins is the subject of the following experiments.

Methods

Quick's One-stage Prothrombin Time.—Quick's one-stage prothrombin times were carried out using " difco " rabbit brain thromboplastin.

Russell's Viper Venom Modification.—Oxalated plasma, 0.1 ml., and 0.1 ml. of 1 in 20,000 solution of " stypven " brand of Russell's viper venom were incubated at 37° C. Then 0.1 ml. of 0.025 M. solution of calcium chloride was added and the time recorded for fibrin particles to appear.

Factor VII.—Factor VII was obtained from normal serum by the addition of an equal quantity of 0.008 M. tricalcium phosphate suspension and the adsorbed Factor VII was obtained in solution by elution of the precipitate with 0.2 M. sodium citrate in a volume one-tenth of the original serum.

$Ca_3(PO_4)_2$-plasma.—$Ca_3(PO_4)_2$-plasma was obtained by centrifuging normal oxalated plasma that had been treated by an equal quantity of 0.008 M. tricalcium phosphate.

Experimental Results

Effect of Russell's Viper Venom (Stypven) on Tromexan Plasma.—It has been shown by Hunter and Tudhope (1953) that the main action of the anti-coagulant drug " tromexan " is to depress Factor VII and not prothrombin, at least in the initial period of treatment. Prothrombin times were carried out on tromexan plasma using " difco " and " stypven " thromboplastins in parallel, and the results expressed as " prothrombin " percentage by reference to saline dilution curves. Plasmas were investigated on different days after the beginning of therapy as shown in Table I.

TABLE I

ONE-STAGE PROTHROMBIN TEST ON TROMEXAN PLASMA

	Day	2	3	4	5	8	11	16
Prothrombin (%) ..	Difco	23	12	34	36	18	19	16
	Stypven	100	33	75	80	55	29	14·5

It can be seen that much higher values are given with " stypven " than with " difco," especially in the earlier period of therapy. The closer approximation of the two methods in the later days may be due to the fact that prothrombin also is reduced if tromexan is continued for this length of time.

Hunter and Tudhope (1953) have shown that normal serum containing Factor VII has an accelerator action on tromexan plasma and the following experiment shows that "stypven" possesses similar accelerator activity. Tromexan plasma was mixed with stypven solution, 1 in 20,000, in the proportion of 9 to 1 and prothrombin times were carried out by Quick's method on successive days of therapy (Table II).

TABLE II

ACCELERATOR ACTION OF STYPVEN ON TROMEXAN PLASMA

Day:	0	1	2	3	5	6	7
Prothrombin time in secs. (Quick):							
Tromexan plasma ..	12	16·5	27	20	21	21	20
90% tromexan plasma + 10% "stypven"	8	8·5	9·5	9·5	10	9·5	12·5

The powerful accelerator action produced by 10% of 1 in 20,000 solution of "stypven" is again most marked in the earlier days of therapy.

Effect of Stypven on Plasma of Congenital Factor VII Deficiency.—The case of congenital Factor VII deficiency used in these experiments has been fully described elsewhere (Jenkins, 1954).

One-stage prothrombin times were measured on several occasions on plasma from this case using "difco" and "stypven" in parallel (Table III). For purposes of comparison the prothrombin times are expressed as "prothrombin" percentage by reference to dilution curves, although it has been shown by the two-stage prothrombin test that in reality only Factor VII is deficient.

TABLE III

ONE-STAGE PROTHROMBIN TEST ON PLASMA OF CONGENITAL FACTOR VII DEFICIENCY

Prothrombin (%) ..	Difco	18	20	15	20	9
	Stypven	83	87	100	78	100

It will be seen that "stypven" gives no indication of Factor VII deficiency and, in fact, the prothrombin values given by "stypven" corresponded closely to those obtained by the two-stage test in this case.

The corrective action of "stypven" on the prothrombin time was demonstrated by mixing the patient's plasma with "stypven" in the proportion of 9 to 1 and measuring Quick's prothrombin times. The results were compared with the corrective effect of similar amounts of normal serum and normal plasma, both of which contain Factor VII or its precursor (Table IV).

The powerful effect of a 1 in 20,000 solution of "stypven" is shown and even in a dilution of 1 in 200,000 the action is comparable with normal serum and greater than normal plasma in correcting the prothrombin time of Factor VII-deficient plasma.

TABLE IV

ACCELERATOR ACTION OF STYPVEN ON PLASMA FROM CONGENITAL FACTOR VII DEFICIENCY

Mixtures (Parts)

Patient's Plasma	Stypven 1/20,000	Stypven 1/200,000	Normal Serum	Normal Plasma	Prothrombin Time (sec.)
				1	17
1					55
9	1				10
9		1			19
9			1		17
9				1	23

Comparison of the Properties of "Stypven Accelerator" and Factor VII.—Factor VII is completely absorbed from serum by $Ca_3(PO_4)_2$ and can be recovered by elution with sodium citrate. Stypven in 1 in 20,000 solution was treated in a similar manner in order to discover whether its accelerator activity also possessed these properties.

The $Ca_3(PO_4)_2$-treated serum, the $Ca_3(PO_4)_2$ "stypven," the eluted Factor VII, and the eluted "stypven" accelerator were each tested in turn for accelerator activity. For this purpose a 10% dilution of normal plasma in $Ca_3(PO_4)_2$-treated plasma was made, thereby ensuring an excess of Factor V, and this was mixed with 10% of the solution to be tested. Prothrombin times were measured in the usual manner and the results are shown in Table V.

TABLE V

COMPARISON OF STYPVEN ACCELERATOR AND FACTOR VII

Mixtures (Parts)

Normal Plasma	$Ca_3(PO_4)_2$ Plasma	Factor VII from Serum	Accelerator from Stypven	$Ca_3(PO_4)_2$ Serum	$Ca_3(PO_4)_2$ Stypven	Prothrombin Time (sec.)
1	9					28
1	8	1				16
1	8		1			14
1	8			1		29
1	8				1	23

It can be seen that the accelerator factor of "stypven" can be adsorbed by $Ca_3(PO_4)_2$ and eluted by sodium citrate in a similar manner to Factor VII except that when a 1 in 20,000 solution of "stypven" is treated with the same amount of $Ca_3(PO_4)_2$ as is used for serum, the adsorption of accelerator from "stypven" is not quite as complete as that of Factor VII from serum.

Discussion

These experiments show that Russell's viper venom has a powerful accelerator action similar in many respects to that of Factor VII and in view of their

widely different origins this close similarity is somewhat remarkable.

It is now believed that the action of Factor VII in blood coagulation is concerned with the formation of plasma thromboplastin (Biggs, Douglas, and Macfarlane, 1953) and it appears to be identical with the "co-thromboplastin" described by Mann and Hurn (1951).

Brain thromboplastin requires Factor VII for its action, whereas Russell's viper venom is more complete in its thromboplastic activity because of the co-thromboplastin or accelerator which it contains. This property explains the powerful haemostatic action of venom but renders it insensitive to the reduction of Factor VII which occurs after the administration of dicoumarol or tromexan. For this reason, "stypven" is useless in the control of treatment with these drugs.

Since rabbit brain extract and viper venom differ in this manner, it is possible that the divergent results obtained by various workers using other sources of thromboplastin, such as human placenta or ox lung, are related to the nature or quantity of the co-thromboplastin factors in these tissue extracts.

The action of rabbit brain extract is the best known of all these tissue preparations and for this reason it is to be preferred for general use.

Summary

Russell's viper venom is shown to have a powerful accelerator action as part of its thromboplastic activity.

The accelerator is closely similar to Factor VII and is probably a co-thromboplastin.

Differences in the thromboplastic activity of other tissue extracts may be related to the co-thromboplastin present.

I wish to thank Dr. H. F. Brewer for his encouragement while carrying out this work.

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TEMPERATURE LEVELS IN HOT-AIR OVENS

BY

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During the past few years the popularity of hot-air ovens for sterilizing has been increasing. This is partly due to the ease with which they sterilize small objects such as syringes, and partly because they are inexpensive to run. At the same time autoclaves, which appear to be popular in America, proved to be difficult to obtain in this country after the war, and were often large and cumbersome; they also required skilled handling. The introduction of syringe services has led to increasing numbers of hot-air ovens being used for sterilization, and it was primarily because of the formation of a syringe service in this area that the present investigations were undertaken.

Aim of the Investigation

Our preliminary investigations were made to assess:

(1) Whether the heat actually penetrated to the syringes. (2) Whether the type of packing employed was satisfactory. (3) At what position in the oven should the thermocouple of an automatic recording apparatus be placed.

Normally it has been the practice to test an oven with thermocouples placed in glass tubes while the oven is empty. The efficiency of the oven is gauged by the degree of correlation of the temperature levels recorded throughout the oven. In our investigations the ovens were tested fully loaded just as in a syringe service, since it is only when heat penetrates the centre of the syringes that sterilization is effective. In the literature opinion varies considerably as to what the temperature of the oven should be and the time required to ensure adequate sterilization. Temperatures varying from approximately 140° C. to 180° C. from half an hour to two hours are mentioned. However, it is probable that in this country 160° C. for one hour, as advocated by the Medical Research Council Memorandum No. 15 (1945) on the sterilization of syringes, would be accepted in a court of law. This memorandum further recommends that ovens should be loaded cold and allowed to heat up to 160° C. and held at this temperature for one hour. In our investigation this temperature has been used in all the tests undertaken.

Recommendations in the Literature

Although experience with hot-air sterilization dates back as far as 1881, Koch and Wolfhügel (1881) found that dry heat was not as effective as wet heat in destroying bacteria and spores, and that, although vegetative bacteria could be destroyed at a temperature of just over 100° C. for one and a half hours, spores required a temperature of 140° C. for three hours. The type of hot-air oven is, however, not mentioned, and it is probable that these were of the gas type.

Since then a number of sterilizing temperature levels have been advocated, and, in view of the

TABLE I

TEMPERATURE LEVELS FOR HOT-AIR OVENS

Name	Hot-air Sterilizer
Bigger, J. W. (1949)	160° C. for 1 hour 140° C. for 3 hours
British Pharmaceutical Codex (1949)	150° C. for 1 hour
British Veterinary Codex (1953)	150° C. for 1 hour
Burrows, W. (1949)	170° C. for 2 hours
American Public Health Association (1950)	160° C.–180° C. for 2 hours
Dubos, R. J. (1948)	160° C. for 90 minutes
Fairbrother, R. W. (1953)	160° C. for ¾–1 hour
Gerhards, G. A. (1952)	140° C. for 1 hour (electric fan oven)
Gradwohl, R. B. H. (1943)	170° C. for 2 hours (gas oven)
M.R.C. Memorandum No. 15 (1951)	160° C. for 1 hour from cold
Mackie, T. J., and McCartney, J. E. (1953)	160° C. for 1 hour 180° C. for ½ hour
McCulloch, E. C. (1945)	160° C. for 1 hour
Smith, D. T., and Martin, D. S. (1948)	150° C.–160° C. for 1 hour
Stitt, E. R., Clough, P. W., and Bradenham, S. E. (1948)	160° C. for 90 minutes (syringes must be separated)
Walter, C. W. (1948)	160° C. for 1 hour if instruments are clean 160° C. for 4 hours if oily or greasy
Whitby, L. E., and Hynes, M. (1951)	160° C. for 1 hour
Wilson, G. S., and Miles, A. A. (1946)	140° C.–160° C. for 1–1½ hours 400° C. for 20–30 seconds
Underwood Weedon, B. (1941)	320° F. for 1 hour (electric oven without fan) (160° C.)

variety of recommendations, have been set out in Table I. In the majority the type of oven has not been specified, but Gradwohl (1948) quotes 170° C. for two hours in a gas oven as being satisfactory. Weedon Underwood (1941) advocates 160° C. for one hour, and illustrates a satisfactory electric oven without a fan. More recently, however, Gerhards (1952) using a fan oven has shown that spores can be satisfactorily destroyed at 140° C. for one hour. Ewald and Schmid (1953) also draw attention to the importance of testing electric ovens with maximum thermometers, and found large variations from shelf to shelf. Walter (1948) in his monograph *The Aseptic Treatment of Wounds* draws attention to the limitation of the hot-air oven, and points out that most ovens heat up slowly and unevenly and that sterilization is therefore uncertain. He advocates that the whole load should reach 160° C. and be held at this temperature for one hour, provided that the instruments are clean and free from oil or grease. If the instruments are oily or greasy he considers four hours after 160° C. satisfactory, but also advocates that "air blowers" in the ovens hasten heating and assure uniform conditions.

Method of Investigation

In our investigations "chance" all-glass metal-tipped syringes were used, and were packed in either thick-walled glass tubes or metal containers. It can be stated now that it was found by experiment that the type of containers used made no appreciable difference to heat penetration or the temperature reached. In the preliminary experiments needles were also used, and these were packed in glass test-tubes with cotton-wool plugs. Throughout the experiments attempts were made to simulate the exact conditions of a normal syringe service. Normally syringes were loaded into wire baskets 9 in. × 9 in. × 9 in. of a type commonly used in bacteriological practice. The baskets were either (1) loosely packed, when syringes in their containers were held apart by metal skewers, leaving a gap of approximately ¼ in. through which air could flow freely, or (2) tightly packed, when as many syringes in their containers as possible were packed in the baskets.

The number of baskets loaded into each oven depended upon the overall capacity. In each case the load was considered as average for the size of the oven. No attempt was made to overload the oven. In two ovens metal trays were provided which did not allow the use of baskets. In these ovens the syringes were laid two deep on their sides to allow the free passage of hot air as indicated in Fig. 1. In selecting ovens care was taken to choose those which were, or might be, in normal use in the laboratory, and were of the standard pattern produced by well-known laboratory furnishers. A multipoint copper

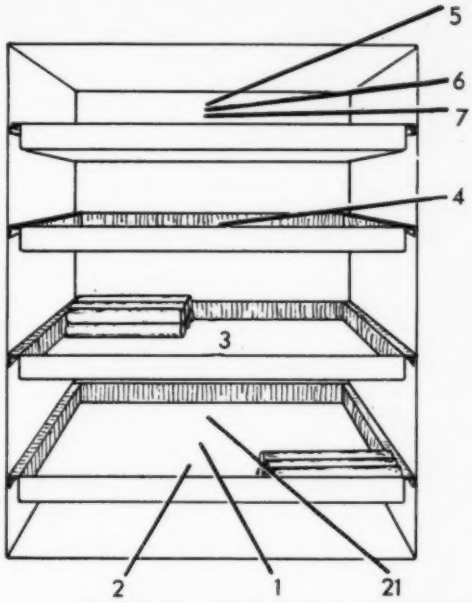

FIG. 1.—The distribution of syringes in electric oven B, packed loosely, 40 syringes to a shelf. The figures indicate the position and number of the thermocouple used.

constant thermocouple temperature indicator was used throughout the experiments. The indication was obtained by a null point method using a potentiometer circuit of high precision, i.e., back E.M.F. was applied against the E.M.F. generated by the thermocouple until a sensitive galvanometer registered zero, and the readings of the potentiometer were calibrated against actual temperatures applied to the thermocouples, which were measured with sensitive thermometers immersed in hot oil. This method cancels out any internal electrical errors in the system.

Specially calibrated mercury and glass thermometers graduated from 100° to 200° C. in half degrees were placed in position as indicated by the manufacturers. The readings from these thermometers were considered to indicate the air temperature of the oven, since it is these readings which guide the operator and are used as a reference point in all experiments except in one electric oven in which the manufacturers had provided a circular-dial type of thermometer.

Special borings were made in the glass piston of some syringes and thermocouples and leads passed into the cavity so that the point rested as close to the base as possible. The syringe was then placed in a container and the lead so arranged that normal packing was not disturbed. The wire leads were introduced into the oven through the hole used for the thermometer. Normally 10 or more leads were used in each experiment, and were distributed in

baskets throughout the oven. Usually two tightly packed baskets and two loosely packed baskets were placed on the top and bottom shelves respectively, and leads placed in the centre of each. Other leads were also placed in a syringe on the outside, or in other baskets. The type of distribution of the baskets and position of thermocouples are shown in Fig. 2. This distribution was varied slightly from oven to oven according to its capacity and the experience gained in previous experiments.

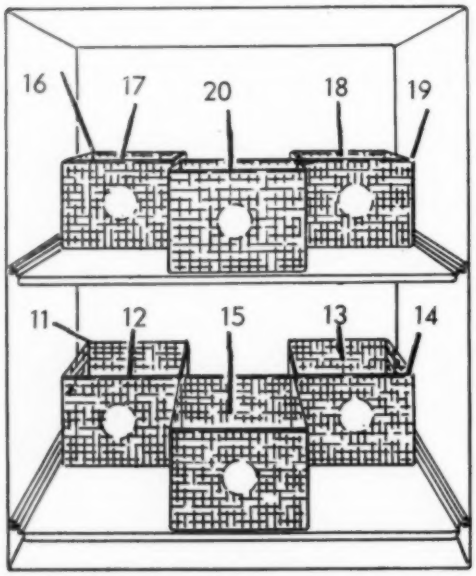

FIG. 2.—The distribution of the thermocouples and baskets used in most of the experiments. The number indicates the number of thermocouple used. Thermocouple: 11 and 16, outside loosely packed syringes. 12 and 17, centre loosely packed syringes. 14 and 19, outside tightly packed syringes. 13 and 18, centre of tightly packed syringes. 15 and 20, centre of loosely packed metal syringes.

In this manner we have tested two gas ovens and four electric ovens without fans and two with fans. With the exception of one gas and one electric oven, all ovens tested conformed to the ±3° C. when tested empty. In the experiments the ovens were loaded cold and allowed to heat to 160° C. and held for one hour at that temperature during which time the temperature recordings were taken at 10 to 20-minute intervals.

Results

Gas Ovens.—In the two gas ovens wide variations of temperature were apparent from the start and generally the temperatures were considerably lower in the bottom shelf than in the top. It was also found that syringes tested near to the back wall were consistently overheated. In the first oven (gas oven A) the reference thermometer reached 160° C. after 50 minutes. At that time the majority of the syringes had not reached 130° C., and differences of approximately 60° C. were noted, if tightly packed syringes are excluded (leads 6 and 9). Most of the syringes on the top shelf seriously overshot the air temperature of the oven, whereas at the end of one hour most of the syringes on the lower shelf, except those placed at the back, had not reached 160° C. Even after one hour there was a variation of 40° C. The recordings of thermocouples are shown in Fig. 3. Similar findings were found in the second gas oven (gas oven B), which also showed some interesting features. This oven had been claimed by its owner as being satisfactory because of the time taken for it to reach 160° C. It was thought that this slow rise would ensure an even distribution of heat. Experiments showed that this oven took 136 minutes to reach 160° C. At this time six out of the 10 thermocouples were below 155° C., the lowest recording being 146° C. The remaining four were at temperatures varying from 166° C. to 182° C. However, during the next 15 minutes the oven air temperature had risen to 163° C. and the syringe temperature varied from 152° C. to 192° C. At the end of one hour six of the syringes were between 193° C. and 169° C., while one syringe still had not reached 160° C., giving an overall variation of 34° C.

Electric Ovens.—The four electric ovens all showed improved performance over the gas ovens. In one electric oven there was only 9° C. difference in the recorded temperature of the syringes at the end of one sterilizing period. In the other three the overall variations of the temperature at the conclusion of the sterilizing period were 21° C., 28° C., and 17° C. if the tightly packed syringes are excluded. This in itself might prevent certain syringes from reaching a temperature necessary to ensure bacterial death. Perhaps the greatest disadvantage of the electric oven without fan is the time taken for the syringes to take up heat. Even with electric oven C, in which the final overall variation was less than 9° C., heat was slowly taken up. For example, although the temperature of the oven reached 160° C. in 63 minutes (and no tight packing was used), the syringes at this time varied from 122° C. to 150° C. (32° C.), and even after 15 minutes there was a variation of 25° C., the temperature of one syringe being only 126° C. It was not until three-quarters of the sterilization time had been completed that the temperature of the syringes became stabilized. These recordings are shown on Fig 4. This oven

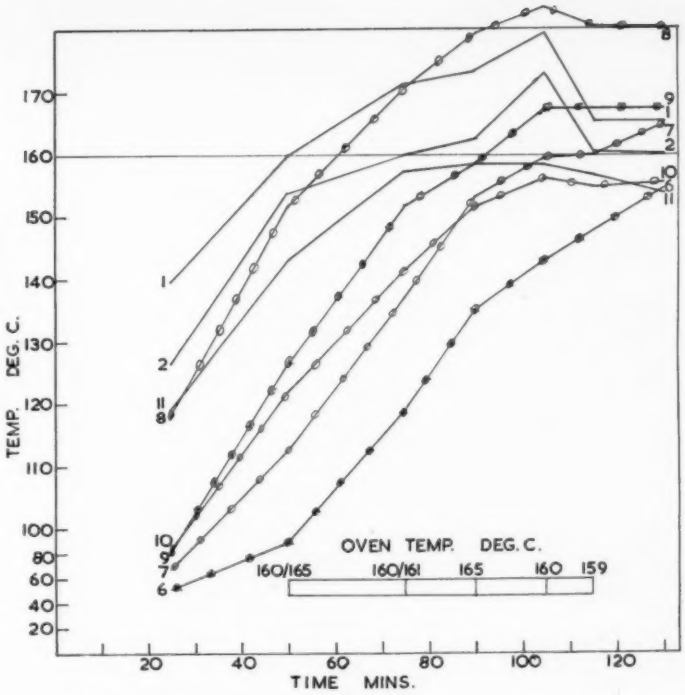

FIG. 3.—Serial temperature recordings of syringes in gas oven A started from cold. Syringes Nos. 1 and 8 were placed in the back of the oven on the bottom and top shelf respectively. O denotes those syringes placed on the bottom shelf. X denotes those placed in the centre of tightly packed baskets. Note the wide variation of temperature and the length of time taken for syringes to attain the temperature of the oven.

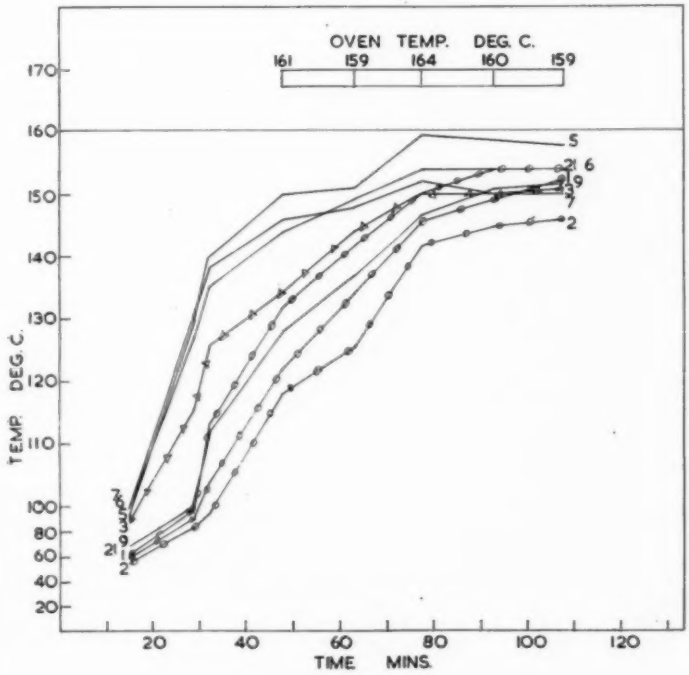

FIG. 4.—Records of thermocouples placed in syringes in gas oven C. O denotes those placed in the bottom shelf, and △ denotes those in the centre shelf.

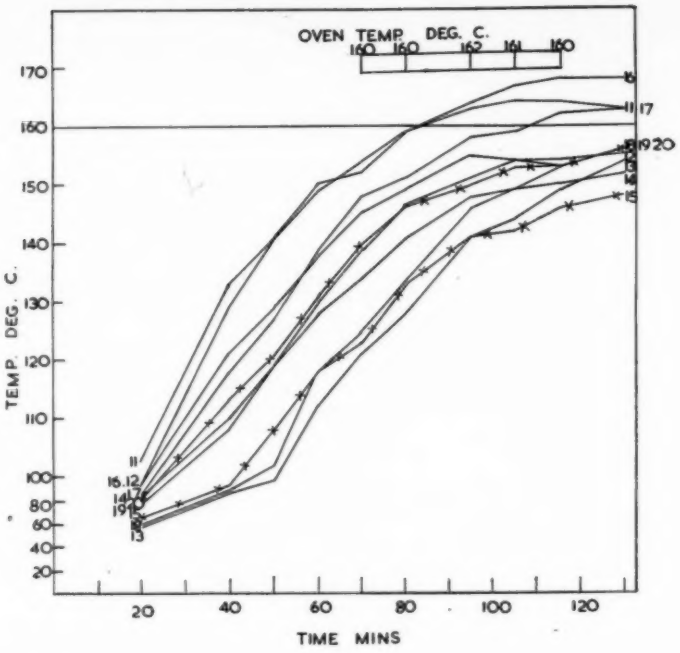

FIG. 5.—Thermocouple reading from electric oven D. Note the slow rise of temperature and the wide distribution of temperature at the end of one hour's sterilization.

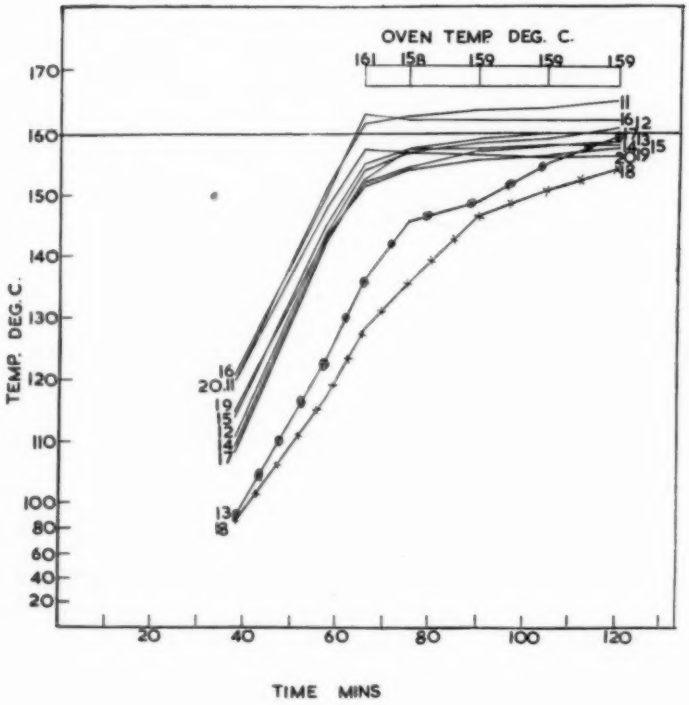

FIG. 6.—Thermocouple readings in syringes, the distribution of which is shown in Fig. 2. Note the prompt rise in temperature but that those in the centre of tightly packed syringes fail to take up heat satisfactorily. X denotes centre of tightly packed syringes. O denotes centre of tightly packed syringes on the bottom shelf.

was fitted with four shelves and a built-in circular-dial type thermometer. It is probable that the dial in this case was faulty and by its correction the temperature of the syringes could have been maintained in the neighbourhood of 160° C. It would not, however, have altered the length of time necessary to bring the syringes up to a stabilizing temperature. It also points out the necessity of checking the recording temperature with that attained by the syringes. Fig. 5 shows the recording in electric oven D, which again shows the delay in the time taken for the syringes to take up heat, and suggests that there is a danger that some of the syringes may not receive sufficient heat to ensure sterilization. Electric ovens A and B showed exactly similar trends.

Fan Ovens.—Two different patterns of electric oven with fans were tested, and it was interesting to note that, although the final overall variation of temperature in the syringes was less than 8° C. and 10° C., the tightly packed syringes again showed a serious delay in taking up heat. In these ovens heat was promptly taken up as shown in electric oven B with fan (Fig. 6), and the overall variation of syringe temperature was less than 12° C. and 13° C. from the start of the sterilizing period, the lowest temperature of any syringe recorded, excluding tightly packed syringes, being 144° C. This ensures that all syringes have similar heat treatment.

Overloaded Ovens.—In all experiments so far quoted, there was a delay in the time taken for the packed syringes to take up the oven temperature. It was therefore decided to test specifically the effect of tightly packed syringes and at the same time using syringes of varying sizes. For this experiment electric oven A, with fan, was used, since it appeared to be the most efficient oven (Fig. 7) tested at that time. The recorded temperatures of thermocouples are shown in Fig. 8 and should be compared with Figs. 3, 7, and 9. It is seen that the syringes in the centre of the tightly packed baskets take a considerable time to reach the required temperature, and further that the overloading of the oven interferes with the normal air flow with the result that many of the syringes fail to reach oven temperature. In general the bottom shelf shows a lower temperature, confirming the interference with air flow which is normally higher in ovens with fans.

Heat-loaded Ovens.—The Medical Research Council Memorandum No. 15 (1945) specifically makes the point that ovens should be loaded cold and the oven allowed to attain the temperature of 160° C., when it should then be held at this temperature for one hour. However, in a busy syringe service considerable time may be wasted to allow the oven to cool to room temperature between batches. It was therefore decided to test specifically the effect of loading the oven when still hot. For this purpose the ovens were allowed to heat to 160° C., and as soon as that temperature was reached the ovens were opened and while they were still hot were loaded with syringes already placed in baskets. The ovens were then allowed to regain the temperature of 160° C. and held for one hour. Two ovens were tested in this way, gas oven A and electric oven A with fan. The distribution of syringes and thermocouples was exactly similar. The temperature recordings are shown in Figs. 8 and 9 and should be compared with Figs. 3 and 6. No appreciable difference is seen in gas oven A, while in electric oven A with fan the uptake of heat is more even and complete, presumably because heat has already been absorbed into the fabric of the oven.

Discussion

It must be clearly understood that the placing of thermocouples enclosed in syringes and shielded by containers are all variables which contribute to the variations in temperature shown earlier. The arrangement of the basket also plays an important part in interfering with hot air currents. The experiments must therefore be considered as indicating trends of temperature changes rather than definitive alterations. Nevertheless, in constructing any piece of sterilizing apparatus it is essential to ensure that all articles receive similar heat treatment, and that the temperature reached corresponds to that of the recording thermometer, reaching this temperature with a minimum of delay. With the exception of fan ovens the majority of ovens do not meet these criteria. In addition, our experiments again stress the importance of avoiding tight packing, since it is clear that by tight packing it is possible that some syringes may receive insufficient heat treatment and may not reach the thermal death point of bacteria. There must be a free flow of air around all articles to be sterilized, and enclosing syringes in metal boxes or similar sealed containers may prevent the heat penetrating to those syringes near the centre. It is also clear that overloading ovens may seriously interfere with heat distribution, a point which has been frequently stressed in the American literature but which does not seem to have had sufficient emphasis in Britain. Maxwell Savage (1936) has also shown that tight packing

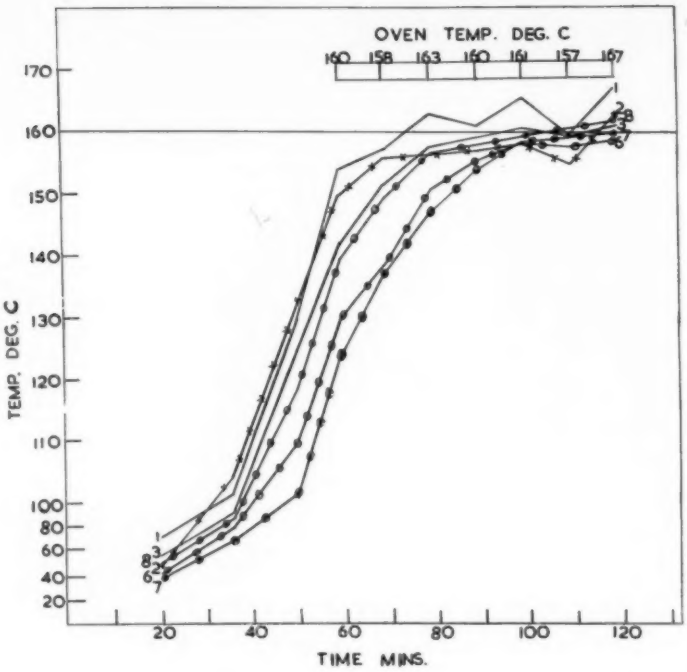

FIG. 7.—Reading of electric oven A with fan. X denotes those placed in syringes in the centre of tightly packed syringes. O denotes those placed in lower shelf. In the final stages the oven was allowed to overheat, and the rapid take-up of heat should be noted.

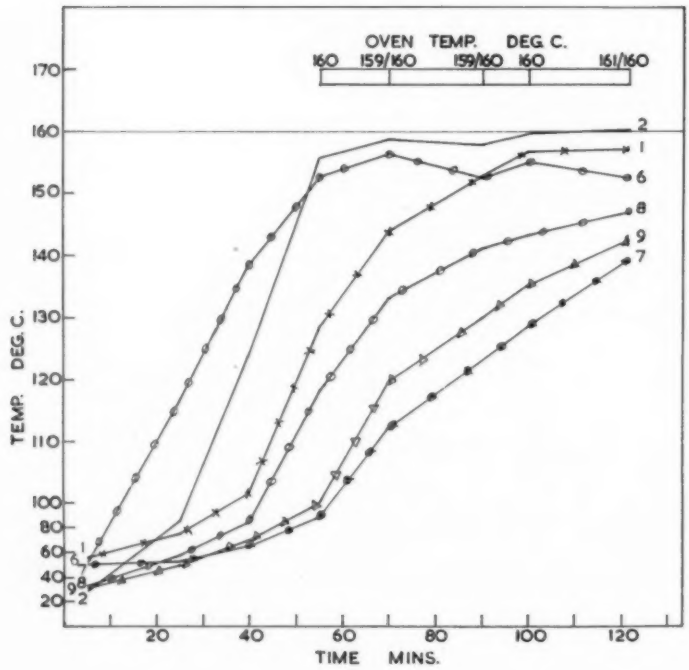

FIG. 8.—Effect of overloading an oven in which all the baskets contain tightly packed syringes. X denotes centre of the basket. O denotes bottom shelf. △ denotes centre of tightly packed 20 ml. syringes.

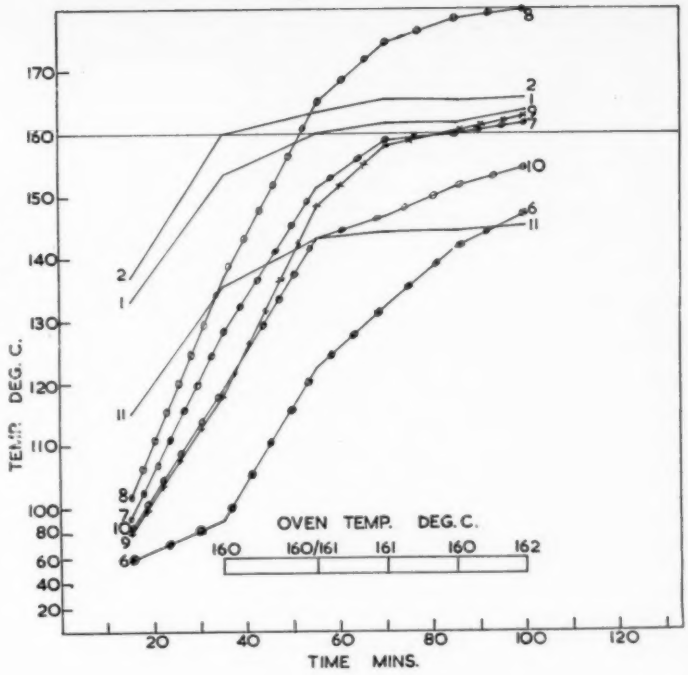

FIG. 9.—This should be compared with Fig. 3 (gas oven A). The same oven position of syringes and baskets, number of thermocouples, and symbols as those used in Fig. 1, the only difference being that the oven was first heated to 160° C. and loaded hot and allowed to regain the temperature of 160° C. before the sterilization procedure was restarted. Note the time saved by this method, and that the uptake of heat and variation of temperatures recorded are similar to that loaded cold.

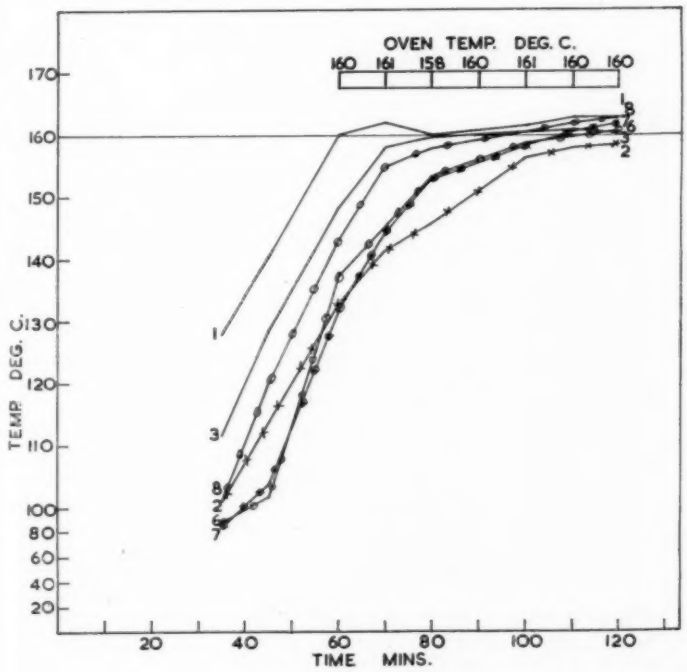

FIG. 10.—The effect of loading electric oven A with fan while it is still hot. Note that the take-up of heat is slightly more rapid and that the overall variation of temperature tends to be less.

is equally dangerous in autoclaves and it is essential that steam should penetrate to all articles to be sterilized.

The uneven distribution of temperatures recorded in gas ovens suggests that this type of oven is unsuitable and unreliable for syringe services. Not only is there a danger that heat may not penetrate, but also there is a possibility that excess of heat may cause the solder to disintegrate if glass and metal syringes are used. A further danger, shown by Gerhards (1952), is that the point of the needle may be seriously damaged by extremes of temperature.

In the electric oven without fan the overall variation of temperature was not so great as with a gas oven. However, without fans the transfer of heat is slow, and variations even after stabilization may be extreme and suggest that the heat treatment may be of such short duration and of such low temperature that the thermal death point of bacteria may never be reached. It is therefore important that in the electric oven the time of sterilization must be so adjusted that the instrument showing the lowest temperature receives adequate heat treatment. This may mean that some articles receive more heat and for a longer period than is necessary. In a busy syringe service when ovens are in constant use the time may be of importance. The value of the fan oven is that the load takes up the heat rapidly and equally from the time the oven reaches the desired temperature and that the variation of temperature from one syringe to another is less than 10° C. This indicates that in a fan oven the temperature need not be so high nor the heat treatment so prolonged, both valuable assets in a busy syringe service.

The belief that a slow rise in temperature is necessary to ensure that heat is taken up evenly is discounted by experiences in gas oven B, for in this experiment 136 minutes elapsed before the oven reached 160° C., and even then a wide variation of temperature was recorded. The experiments of loading the oven while hot indicate that the heat is taken up more evenly and more quickly. This is probably due to the fact that the fabric of the oven has already absorbed heat and allows a greater and more even transfer to the articles to be sterilized. The fact that so many of the ovens showed a satisfactory distribution of heat when empty suggests that perhaps more rigorous tests should be employed by the manufacturers before issuing ovens for distribution. It is suggested that probably a standard load when testing might be necessary to ensure an adequate margin of safety.

No attempt has been made in this investigation to determine the thermal death point of bacteria usually encountered. Experiments with spores with the oven at 160° C. for one hour seemed satisfactory. However, no record of temperatures after loading seems to have been carried out, and it is probable, in accordance with Gerhards' paper (1952) on the thermal death point of bacteria in fan ovens which stated that 140° C. for one hour was sufficient, that this temperature is in fact effective, and it is assumed that the reason why so many authoritative manuals have specified a higher temperature is because in the past the wide variation of temperature within gas and electric ovens necessitated this precaution.

The organization of syringe services demands simplified cleansing and sterilizing procedures. The fact that in autoclaves syringes should be sterilized unassembled to ensure adequate steam penetration makes this method clumsy, since it requires two further steps. The syringes must be reassembled after sterilization and the entrance holes to the containers closed. Hot-air sterilization is therefore preferable, since it avoids both these extra operations. Preliminary experiments with thermocouples with syringes during the process of boiling show that even after three minutes the syringe is not yet at the temperature of 100° C. and that by wrapping in lint heat penetration is prevented for five minutes or longer, and proves that little reliance can be placed on this method of sterilization. The work of Bigger, Blacklock, and Parrish (1940) proves conclusively that even after 20 minutes not all bacteria may be destroyed, and this is accentuated if any biological material adheres to the syringe. Removing adherent biological fluids such as pus or blood together with possible spores is one of the most important preliminary steps in the sterilizing process. Once this has been done, a standard period of dry heat treatment will ensure adequate sterilization.

Spores are known to be most resistant of all organisms to dry heat. They therefore suggest themselves as suitable test organisms, but they too vary in their resistance to heat. Unfortunately most authors do not indicate the precise organisms used to test the reliability of their ovens, and it is possible that this omission accounts for some of the discrepancies in the literature. For this reason it is essential to carry out further work on the thermal death-point of organisms in the presence of biological fluids with special reference to the length of time and the temperature necessary to ensure sterilization. An authoritative body should define the organisms to be used for testing dry heat ovens.

Summary

An investigation with multipoint thermocouples into loaded ovens has been carried out. Two gas ovens showed a wide variation of temperature, which makes these instruments unsatisfactory for syringe services. Experiments with four electric ovens without fans showed that in the main heat is taken up slowly and that, even though close correlation of temperature is obtained when the oven is empty, wide variations of temperature may occur. In the fan oven heat is promptly absorbed and the variation of temperature is reduced. Loading ovens while still hot does not interfere with the transfer of heat to the articles to be treated, and in fact in the majority of instances it is to be preferred, but tight packing of syringes and needles is condemned as unreliable.

Our thanks are due to the directors of Messrs. Townson & Mercer for the loan of apparatus, to Mr. W. H. R. Saunders for assistance in the technical work, and to Mrs. W. Tuke, S.R.N., for general assistance.

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THE CLASSIFICATION OF ANAEROBIC COCCI AND THEIR ISOLATION IN NORMAL HUMAN BEINGS AND PATHOLOGICAL PROCESSES

BY

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In a previous paper (Hare, Wildy, Billett, and Twort, 1952) a scheme employing fermentation of carbohydrates and organic acids was put forward for the classification into six groups of the anaerobic cocci derived from human beings.

There is at present extremely little information as to the occurrence of anaerobic cocci on the mucous and skin surfaces of normal human beings. This, together with a study of strains from a number of infective processes, forms the subject of this paper.

Material and Methods

Source of Strains.—The following were chosen.

Nose.—Woollen swabs on straight applicators were employed to obtain samples from the anterior nares of 54 normal persons, medical students and technicians, during the first three months of 1953.

Throat.—Swabs from the tonsillar region of 15 normals were examined during the first six months of 1953.

Tonsils.—Single tonsils from 80 persons, mostly children, were washed with sterile saline immediately after excision. They were seared on the lateral surface, incised from this aspect with a sterile knife, and samples obtained with a platinum loop.

Faeces.—Stools were obtained from 50 in-patients, mostly young adults, suffering from some condition in which the alimentary tract was not considered to be involved.

Vagina.—High vaginal swabs were taken during the first three months of pregnancy from two series of patients, the first of 48 persons during the summer of 1948, and the second of 45 persons during the summer of 1953.

Skin.—Swabs moistened with sterile broth were used to take samples from the dorsum of the right wrist of nine persons.

Gums.—A platinum loop was used to take samples from the gum margin in the region of a lower canine tooth from nine persons with apparently healthy gums.

External Auditory Meatus.—Dry swabs on straight applicators were used to investigate nine persons.

Strains from Infections.—Three groups of infections were chosen.

Puerperal Pyrexia.—Eight strains from cases in this hospital, 48 isolated by Dr. R. M. Calman, two by Dr. L. R. Janes, one by Dr. A. Beck, and one by Dr. A. Cunliffe were examined. No patient was severely infected.

Infections of the Respiratory Tract.—Strains from 16 cases of chronic bronchitis, bronchiectasis, lobar pneumonia, asthma, pulmonary collapse and empyema, from four abscesses of the lung removed by lobectomy, and from 14 specimens of fluid obtained during bronchoscopy in suspected cases of carcinoma of the lung were sent us by Dr. Weatherley Mien and Mr. N. R. Barrett.

Other Infections.—Strains from cases with an infected frontal sinus, gangrenous appendix, appendix abscess, sinus following appendicitis, brain abscess, operation wound, infection of the foot, and two from the blood in bacteraemia were sent us by Dr. G. Martyn, Dr. Anne Gibson, Dr. L. R. Janes, Dr. J. Bamforth and Dr. R. M. Fry, Dr. J. Pinniger and Dr. H. Loewenthal.

Media for Isolation of Strains from Normal Persons.—Previous experience had shown that a suitable solid medium for the cultivation of anaerobic cocci contained Evans peptone 2.0%, Difco yeast extract 0.2%, agar 2.0%, and 5% horse blood. Since sodium oleate, 0.01%, has no inhibitory effect on these organisms but may promote the growth of strains of Groups III and IV, it was added to the medium (Wildy and Hare, 1953).

Although many specimens were plated in parallel on the same medium containing 40 units per ml. Polymyxin E to inhibit Gram-negative bacilli

(previous work having shown that at this concentration the antibiotic has no effect on the anaerobic cocci (Hare *et al.*, 1952)), there was no evidence that anything was gained by this procedure.

Isolation of Strains.—Whenever possible the specimens from normal persons were plated within a few minutes of being taken. The plates were incubated anaerobically for 48 hours in a McIntosh and Fildes jar. Subcultures of each variety of colony found on such plates were made in parallel on two blood agar plates, one of which was incubated anaerobically and the other aerobically. After 48 hours Gram-stained films were made of those strains growing on the anaerobic plate, but which failed to grow on the aerobic plate. Cocci isolated in this way, irrespective of their Gram reaction, were subcultured twice more from single colonies on to plates incubated anaerobically and aerobically. Provided there was no growth on the plates incubated aerobically on the three occasions, the strain was accepted as an anaerobic coccus and preserved by transferring a fairly heavy inoculum to Robertson's meat medium, incubating for 24 hours, and then storing in the refrigerator.

Many of the strains from patients with infections were isolated by other workers on their routine media. Those isolated in this hospital were obtained from blood agar plates made with Lemco broth. Care was taken on receipt of such strains to determine their purity and that they fulfilled our criteria in regard to anaerobiosis.

Classification of the Organisms Isolated

In the original scheme put forward by Hare *et al.* (1952) fermentation of five carbohydrates (glucose, fructose, maltose, sucrose, and galactose) and five organic acids (pyruvic, lactic, citric, malic, and tartaric) was employed, but in a subsequent paper Wildy and Hare (1953) showed that, although 0.01% sodium oleate had no detectable effects on Groups I, II, V, and VI, strains of Groups III and IV could ferment one or other substance much more actively in its presence. Hare *et al.* (1952) also showed that the presence of a sulphur compound was essential for the production of gas by Group I strains.

For these reasons the basal medium employed for classification contained 0.01% sodium oleate and 0.1% sodium thioglycollate in 2% Evans peptone, with 0.2% Difco yeast extract.

Each batch was made in the same way, the peptone, yeast extract, and sodium thioglycollate being dissolved in distilled water, brought to the boil, and filtered through hard filter paper at once. Sodium oleate, 0.01%, was then added. To this medium 1% of one or other of the five carbohydrates or the sodium salts of lactic, tartaric, and citric acids were added. Because the sodium salts of pyruvic and sodium malic acids were not available, the acids themselves in concentrations of 1% were added. The *p*H of the medium was adjusted to 8.0 and 2.5 ml. placed in 5 in. × ½ in. tubes. Melted vaseline was run on the surface and the tubes sterilized by steaming on three successive days. The same medium containing 1% pyruvic acid but no added oleate was employed for growth of the strains before inoculation into the test media.

Of the 233 strains referred to in this paper, 63 had been classified by Hare *et al.* (1952) by the same methods but in media without added oleate. These strains were not retested in the media described above, but there is no reason to suppose that different results would have been obtained, since the very great majority were members of groups unaffected by fatty acid. The remaining 170 strains were all tested in oleate-containing media. For this purpose about 0.1 ml. of a 48-hour culture in the inoculating medium described above was inoculated into each of the test media. The cultures were incubated for seven days. The length of gas column produced by displacement of the vaseline plugs upwards was measured, the opacity of the culture on the Brown and Kirwan (1915) scale ascertained, and the *p*H determined with indicators on porcelain tiles. Representative strains from each of the groups were included in any series of tests with new batches of media. These tests showed that, provided the media be made in the same way, different batches behave in an almost identical manner.

If fermentation of the carbohydrates occurred the *p*H was depressed from the initial 7.6–7.8 to 5.6–6.4, and if gas was formed the vaseline plug was displaced upwards, usually by 10 mm. or more. When the organic acids fermented the *p*H was seldom depressed very much, but large quantities of gas were produced, and it was on this feature that ability to ferment organic acids was assessed.

Deep cultures of all the strains were made in 1.5% plain agar in broth containing 2.0% peptone, 0.2% yeast extract, 1.0% pyruvic acid, and 0.01% sodium oleate, and the distance between the surface and the upper edge of growth measured, in order to provide some data as to the degree of anaerobiosis required by each strain.

Films were also made from the inoculating culture, stained by Gram's method, and the size and arrangement of the cocci of each strain ascertained.

Results

Table I summarizes the behaviour of members of each of the groups in the oleate-sulphur-containing media described above. It will, however, be observed that in addition to Groups I–VI, already described by Hare *et al.* (1952), three more groups have been added. This was necessitated by the fact that a sufficiently large number of

TABLE I
SCHEME OF CLASSIFICATION EMPLOYED

Group	Substrate Employed											Habit of Growth
	Control	Glucose	Sucrose	Maltose	Fructose	Galactose	Pyruvate	Malate	Citrate	Tartrate	Lactate	
I (*Strep. putridus*)	—	AG	—	AG	AG	—	G	—	—	—	—	Chains
II	—	—	—	—	—	—	G	—	G	G	—	Masses
III	—	AG	AG	AG	AG	AG±	G	—	—	—	—	,,
IV	—	—	—	—	A	—	G±	—	—	—	—	,,
V	—	—	—	—	—	—	G	G	G	G	G	,,
VIa	—	A	A	A	A	A	—	—	—	—	—	Chains
VIb	—	A	A	A	A	A±	—	—	—	—	—	Masses
VII	—	A	—	—	A	—	—	—	—	—	—	Masses and chains
VIII	—	AG	—	—	AG	—	G	—	—	—	—	Masses
IX	—	—	—	—	—	—	—	—	—	—	—	,,

A = acid, G = gas, in media containing 0.01% sodium oleate, 0.1% sodium thioglycollate, and 1% of the substance.

TABLE II
THE PRESENCE AND CLASSIFICATION OF THE ANAEROBIC COCCI IN NORMAL HUMAN BEINGS

Site	No. of Persons Examined	No. of Persons Carrying Anaerobic Cocci	Percentage of Individuals Sampled who were Carriers	No. of Representatives of Each Group	Group										
					I	II	III	IV	V	VIa	VIb	VII	VIII	IX	Not Classified
Nose ..	54	23	42·6	34	0	1	1	8	0	0	3	1	15	0	5
Throat ..	15	10	66·7	12	0	0	0	0	3	1	7	0	0	1	0
Gums ..	9	7	77·8	8	0	0	0	0	6	0	2	0	0	0	0
Tonsils ..	80	38	47·5	43	0	0	0	0	1	5	8	0	0	22	7
Faeces ..	50	5	10·0	6	0	0	0	0	1	0	0	2	0	1	2
Vagina ..	93	22	23·7	29	4	2	7	6	0	0	0	0	1	3	6
Skin ..	9	4	44·4	8	0	0	1	2	0	0	0	1	3	0	1
Ear ..	9	1	11·1	1	0	0	0	0	0	0	1	0	0	0	0
			Total ..	141	4	3	9	16	11	6	21	4	19	27	21

strains having similar characteristics was isolated in the course of the work. Thus, of the 233 strains studied in this way, all but 27 could be placed in one or other of the nine groups.

From Table II it is evident that anaerobic cocci are comparatively common on the mucous and skin surfaces of human beings. They were present in the anterior nares of 42.6% of the population sampled, with Groups IV and VIII predominating. In another series of investigations on 95 nurses, our colleague Dr. N. P. L. Wildy isolated Group IV strains from 31. The throat (surface of the tonsils and posterior wall of the pharynx) was studied in only 15 persons, but 66.4% were carriers, the majority of the strains being Group VI. No less than 38 out of the 80 tonsils examined contained anaerobic cocci, with Groups VI and IX predominating. Only about one in four of these tonsils showed pathological changes to the naked eye. The highest carrier rate of all was found in the examination of the gums, seven out of the nine persons yielding these organisms.

In contrast to the nasopharynx, anaerobic cocci would appear to be unusual in the faeces, for only six strains were isolated from the 50 specimens examined. Only one of these strains was a member of groups previously described (Group V). Although the very low yield may have been due in part to the fact that the medium employed for isolation was only partially inhibitory for the coliforms present and the colonies of anaerobic cocci are not distinctive, very careful search was always made.

The specimens from the vagina were not "normal" in that they were all taken after pregnancy had started, but anaerobic cocci were undoubtedly present. The carrier rate, 23.7%, was somewhat lower than that reported by White (1933) in a previous investigation (30% in the last month of pregnancy), but it is significant that four of the strains were members of Group I (*Strep. putridus*) which is almost certainly the principal cause of puerperal infection by anaerobic cocci.

The surface of the skin of the wrist was studied in only nine persons, but four carried anaerobic cocci. These belonged to groups common in the nasopharynx, suggesting that they come from this area. The external auditory meatus, on the other hand, yielded only one strain.

In specimens from all the areas sampled except the ear, a number of individuals were found to be carriers of cocci of more than one group. An extreme example of this was a sample taken from the skin which gave 12 colonies warranting further study. Two were Group III, three Group IV, two Group VIII, and five could not be allocated to a group but behaved in the same way.

Strains Isolated from Pathological Processes

In addition to the investigation of anaerobic cocci in normal persons, an attempt has been made to ascertain the possible pathological significance of the different groups. The results obtained are given in Table III.

It is evident that, when anaerobic cocci are present *post partum* in the vagina of patients with pyrexia, Group I is by far the commonest variety. Probably because these organisms are sensitive to penicillin, which is used nowadays as soon as pyrexia develops, none of the patients from whom these organisms were isolated were infected with any severity. Nevertheless, very similar organisms have been isolated from severely infected (several fatal) cases by previous workers (Schottmüller, 1910; Harris and Brown, 1929; Colebrook and Hare, 1933; Stone, 1940). But this organism seems to be responsible for other forms of infection besides puerperal fever, since it was also found in an infected wound of the foot, an abscess of the brain, a septic operation wound, and a gangrenous appendix.

Specimens from the respiratory tract, such as material removed at bronchoscopy, lung abscesses removed by operation, and sputa from chronic lung infections, yielded many strains of anaerobic cocci. It is quite impossible to make any definite statement in regard to the role of these organisms in the pathological conditions from which they were derived. There was certainly evidence of bacterial infection, but other species of organisms besides anaerobic cocci were always present. The anaerobic cocci isolated were members of Groups IV, V, and VI, which appear to be common inhabitants of the normal respiratory tract.

Of the remaining strains four, belonging to Group VIa, came from an infected frontal sinus, an appendicular abscess, a sinus following appendicitis, and the blood of a case with ill-defined pyrexia. Another strain (also from ill-defined bacteraemia) could not be allocated to a group.

Morphology of the Anaerobic Cocci

The majority of the cocci isolated were Gram-positive spheres, 0.6 to 0.8 μ in diameter, showing no particular arrangement under the microscope. But strains of Groups I and VIa grow in the form of chains, as do two of the four strains of Group VII. Strains of Group V are also Gram-positive but only in young cultures. After 24 hours they are almost all Gram-negative. The size of the cocci of Groups III and IV depends on the oleate content of the medium, being 0.7 to 0.8 μ when sufficient quantities are present, but in ordinary media without added oleate they tend to be larger, diameters of 1.0 to 1.6 μ being common (Wildy and Hare, 1953).

TABLE III

CLASSIFICATION OF THE ANAEROBIC COCCI FOUND IN DIFFERENT TYPES OF INFECTION

No. of Patients	No. of Patients with Anaerobic Cocci	No. of Representatives of Each Group	Group										
			I	II	III	IV	V	VIa	VIb	VII	VIII	IX	Unclassified
						Respiratory Tract							
						Material Aspirated at Bronchoscopy							
14	9	10*	0	0	0	2	5	2	1	0	0	0	0
						Abscess of Lung Removed by Operation							
4	2	3*	0	0	0	1	1	1	0	0	0	0	0
						Sputum from Chronic Lung Infections							
16	8	10†	0	0	0	3	5	1	1	0	0	0	0
						Genital Tract							
						Puerperal Pyrexia							
		60	42	5	2	3	0	1	2	0	0	0	5
						Other Forms of Infection							
		9	4	0	0	0	0	4	0	0	0	0	1
Total		92	46	5	2	9	11	9	4	0	0	0	6

* One Group V and one Group VIb isolated from the same specimen. † One patient with Group V and Group VIa; one patient with Group IV and Group VIa.

Degree of Anaerobiosis Required for Growth

Employing deep cultures in agar, measurement of the distance between its surface and the upper edge of growth gave indications of the degree of oxygen tolerance of each strain. In general, this distance was 5–15 mm. and with many strains has remained relatively constant for several years. Contrary to what is usually supposed, very few strains have become capable of aerobic growth with continued subcultivation.

Discussion

Although gas formation, fermentation reactions, and morphology have been suggested by previous workers (notably Prévot, 1948) as suitable criteria for the classification of the anaerobic cocci, attempts to use these tests have generally come to nothing, for it is quite frequently impossible to obtain regular results or to detect any metabolic activity whatever. This is undoubtedly due to the rather peculiar requirements of these organisms, sulphur compounds being required for gas formation by some strains and fatty acids for fermentation by other strains, while only organic acids and not carbohydrates can be attacked by still other strains. There seems no doubt that sufficient amounts of some of these substances may be present as impurities in some batches and absent in other batches of media to nullify the value of any tests in which peptone, agar, or digest broth are employed for the growth of these organisms. But, provided such possible sources of error are borne in mind and care be taken to add the accessory substances known to be necessary, these organisms can not only be classified into nine groups, but members of each of the groups behave in a consistent way when tested in different batches of medium.

It is evident that anaerobic cocci of one or other group are comparatively common commensals in human beings, but that each area tends to possess a rather restricted number of groups. Because they are all sensitive to penicillin, their pathological importance at present is very small. But strains of Group I (*Strep. putridus* Schottmüller) were at one time an important cause of puerperal infection, and this is certainly by far the commonest group found in the puerperal pyrexias occurring nowadays. Group I strains may also occur in non-puerperal forms of suppuration, and from the description of the strains isolated from anaerobic streptococcal myositis by MacLennan (1943) it would appear that some at least are due to Group I strains.

Anaerobic cocci are stated to be common in appendicular abscesses (Altemeier, 1938). We have had no opportunity to verify this, but, of the three strains from infections in this area, one was Group I and two Group VIa. No strains from chronic burrowing ulcer (Meleney and Johnson, 1937), human bite infections (Boland, 1941), and post-operative synergistic gangrene (Brewer and Meleney, 1926) were examined by us. Nor is it possible from the published descriptions to fit the strains isolated by the workers concerned into our classification.

The organisms belonging to Groups II to IX would seem to have less pathological importance. It is, however, possible that, because most of these strains will ferment carbohydrates or organic acids such as pyruvate, they may in some situations so lower the local *p*H that the local defensive mechanisms cannot cope with other organisms which may also be present. In this way they may prepare the ground without necessarily being pathogenic themselves. They may therefore possess more importance than would at first sight appear.

Summary

Anaerobic cocci are frequently present on the mucous and skin surfaces of normal persons.

Employing fermentation reactions in the presence of sodium oleate and sodium thioglycollate, the majority of strains encountered can be assigned to one or other of nine groups.

A comparatively restricted number of groups predominates in the flora at various sites of the human body. There is reason to believe that strains of Group I (*Strep. putridus*) are the commonest potential pathogens.

In addition to the many colleagues mentioned above who have assisted us by sending strains or specimens, our thanks are also due to Dr. van der Merwe, Mr Garland, and Mr. Shaw of this hospital, to Dr. H. J. Parish of the Wellcome Research Laboratories for the Polymyxin E, and to Mr. E. Winslade for technical assistance.

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CLOT CULTURE IN TYPHOID FEVER

BY

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In early diagnosis of typhoid fever, the emphasis has for long been on the isolation of *S. typhi* from blood culture. Clot culture techniques have not enjoyed the same vogue, although they are largely used in areas where the disease is endemic. Felix (1924) found that clot culture was hardly ever unsuccessful if blood culture was successful, but that the reverse was frequently true. He attributed this to the removal of any bactericidal serum factors before culturing the clot.

In an earlier publication (Thomas, Watson, and Hewstone, 1954) we recommended the use of streptokinase bile broth (containing 100 units/ml. of streptokinase) as a culture medium for blood clot. Our experience with this method since then has amply borne out the initial favourable results, and as a result of its application it soon became apparent that the results of clot culture were considerably better than those of blood culture. Thus, in a series of 48 cases where both clot culture and blood culture were performed before the introduction of streptokinase and one or both yielded a positive isolation, there were 38 positive blood cultures and 36 positive clot cultures. In a further series of 99 cases since the introduction of streptokinase there were 48 positive blood cultures and 93 positive clot cultures, an increase of 45 in favour of the latter.

Two possibilities arose that might be responsible for these results: (1) streptokinase might exert a specific stimulating effect on the growth of *S. typhi*, or (2) removal of the serum before culturing the clot increases the chances of obtaining a positive isolation. We have investigated (1) at some length, but have been unable to demonstrate any specific effect on growth, and the action of streptokinase appears to be, as we previously reported, purely an enzyme one concerned with fibrinolysis.

A study was therefore undertaken of the effect of serum factors on the isolation of *S. typhi* from blood clot and whole blood.

Serum Factors in Clot Culture

Preparation of Artificially Infected Clots.—Samples of blood, each of 5 ml., from a healthy donor were added to 1.0 ml. quantities of a saline suspension of a freshly isolated strain of *S. typhi* containing approximately 30 viable organisms as estimated by plate count. The blood–bacterium suspension was thoroughly mixed and the blood allowed to clot. After two hours at room temperature the separated serum was removed. Each clot was then bisected, using a sterile Petri plate and two microscope slides for each, the clot being tipped into the plate, held by the edge of one slide and divided with the other. One half of each plus the total serum volume was incubated in 15 ml. of streptokinase bile broth in a universal container, and the other half clot alone in another 15 ml. Subcultures were plated daily on to Wilson and Blair's medium. Results are detailed in Table I.

TABLE I

Days' Incubation	Clot with Serum		Clot Without Serum	
	Positive	Negative	Positive	Negative
1	17	11	28	Nil
7	17	11	—	—
14	17	11	—	—

After 14 days' incubation 11 of the 28 half-clots incubated with serum gave a negative result, whereas all 28 were positive where half-clots alone were cultured. Since these were corresponding halves of the same clots it is improbable that the former were initially sterile.

When the above method was applied to clots from clinical cases of typhoid fever the results in Table II were obtained in a series of 35 consecutive cases.

Table II shows that nine of the 35, with added serum, gave a negative result after 14 days' incubation where the corresponding half-clot was positive, but only one gave a negative result without added serum where the other half was positive. The chance occurrence of such a result has been estimated at 1 in 93. Analysis of the number of days required to produce a positive result in those cases where both

TABLE II

No. of Cases	Half-clot with Serum		Half-clot Alone	
	No. of Cases	Days' Incubation	No. of Cases	Days' Incubation
35	15	+1	26	+1
	3	+2	5	+2
	4	+3	3	+3
	2	+4	1	−14
	1	+6		
	1	+7		
	9	−14		

+ = Positive isolation. − = Negative result.

halves were positive gives a mean difference of 0.80 ± 0.283 days. The ratio of the mean difference to the standard error is thus 2.83, which is significant. Agglutinin titres were estimated in all the above 35 cases, but were not significantly higher, where a negative result was obtained with added serum, than in those which were positive.

Serum Factors in Blood Culture

A study of the effects of serum factors in blood cultures was performed as follows.

Volumes of 0.5% taurocholate broth, each of 5 ml., were placed in sterile 5 in. by 5/8 in. tubes. To each was added 0.1 ml. of a saline suspension of *S. typhi* containing approximately 25 viable organisms, together with varying volumes of fresh citrated blood from a donor. Tubes were incubated at 37° C. and subcultures plated daily on to Wilson and Blair's medium. Results are shown in Table III.

TABLE III

Volume of Medium (ml.)	Volume of Blood (ml.)	Days' Incubation							
		1	2	3	4	5	6	7	14
5·0	0·5	+	+	+	+	+	+	+	+
5·0	1·0	+	+	+	+	+	+	+	+
5·0	1·5	−	+	+	+	+	+	+	+
5·0	2·0	−	−	−	−	−	−	−	−
5·0	2·5	−	−	−	−	−	−	−	−
5·0	3·0	−	−	−	−	−	−	−	−
5·0	4·0	−	−	−	−	−	−	−	−
5·0	5·0	−	−	−	−	−	−	−	−

+ = Positive isolation. — = Negative result.

Using this particular strain of *S. typhi* (A2), a bactericidal effect was evident where the ratio of blood to medium was less than 1 in 4 to 1 in 5. When the experiment was repeated with six different strains, using approximately the same initial inoculum as above, two resulted in positive isolations where the blood–medium ratio was 1:2, and four gave results similar to those in Table III.

Discussion

Felix (1924), Soman (1932), and Compton and Said (1933) all stress the necessity for removing serum before culturing the residual clot, and the results in Tables I and II support the need for this precaution. Of even more importance in our experience is the need to bring about quick lysis of the clot. In a series of 96 positive clot cultures before the introduction of streptokinase broth, the average period of incubation required to produce a positive result was 6.8 days with a variation of one to 16 days. After the introduction of streptokinase broth the average period required in 170 positive cultures was 1.9 days with a variation of one to four days. Recently we have obtained positive isolations in less than 24 hours by culturing the clots in streptokinase broth in 4 oz. bottles containing Wilson and Blair agar slopes.

It has been our custom to perform blood cultures by adding 8–10 ml. of blood to 35 ml. of 0.5% taurocholate broth, but, in view of the results in Table III, the volume of medium would appear to be too small in relation to the volume of blood. Volumes of culture medium reported in the literature vary considerably; Batty Shaw and Mackay (1951) used 15 ml. of taurocholate broth, whereas Treffers (1948) recommended 190 ml. medium. Pulvertaft (1930) advocated the addition of 10 ml. of blood to at least five times that volume of medium. Where the volume of medium is small the theoretical advantage of adding a large volume of blood to increase the chances of obtaining an infected sample may be outweighed by the disadvantage of increasing the concentration of bactericidal serum factors. Recently we have increased the volume of medium from 35 to 50 ml., and in a series of 30 bacteriologically proved cases of typhoid fever since then there were 23 positive blood cultures, i.e. 77%, and 30 positive clot cultures. This compares with 48 positive blood cultures in a series of 99 cases where the volume of culture medium was 35 ml., i.e., 48.4%. It would seem essential to increase both the volume of medium and the volume of blood if the most satisfactory results are to be obtained.

The failure to find any correlation between the agglutinin titres and the occurrence of positive or negative results of culture in the 35 clinical cases is in keeping with the findings of others. Khairat (1946), for example, could find no correlation between agglutinin titres and the susceptibility of the infecting strain by testing *in vitro*.

Summary

Streptokinase bile broth as a culture medium for blood clot has increased the number of positive isolations and decreased the duration of incubation required compared with ordinary bile broth.

Clot culture techniques, in our experience, have proved superior to the blood culture.

Bactericidal serum factors may exist in sufficient concentration to militate against successful isolation of *S. typhi* where the volume of medium is too small.

No correlation between the bactericidal action of serum and the level of circulating agglutinins was found.

The bactericidal action of serum on *S. typhi* is dependent on the strain of organisms as well as on the serum.

I am indebted to Dr. J. C. Thomas, Provincial Pathologist, Natal Provincial Administration, for his interest, and to the late Dr. G. Bateman, Mathematics Department, University of Natal, for statistical analysis of results.

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A ROUTINE METHOD FOR TESTING THE SULPHONAMIDE SENSITIVITY OF ORGANISMS CAUSING URINARY INFECTIONS

BY

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Several methods are available for testing the sensitivity of organisms to the sulphonamide drugs (Harper and Cawston, 1945 ; Evans, 1948 ; Kokko, 1947), but none of them is entirely suitable for clinical use. The main difficu'ty in using a test similar to that for the antibiotics is the presence of sulphonamide-antagonizing substances in routine media. Such substances were first found in peptone (Lockwood, 1938): it was shown that their effect could be counteracted by human blood, heart broth, and serum (Lockwood, 1938 ; Stamp, 1939). Two lines of approach have therefore been followed in developing sulphonamide sensitivity tests, the first using a synthetic medium free from antagonizer (Kokko, 1947) and the second aiming at their removal from ordinary nutrient media (Harper and Cawston, 1945). Elaborate synthetic media have many disadvantages, and organisms may appear more sensitive than in the body. The removal of antagonizer from ordinary routine media by lysed horse cells, as described by Harper and Cawston (1945) and used by Evans (1948), necessitates careful estimation of the amount required for each batch of peptone ; in most cases a high concentration of blood is needed and in this way false results may be obtained.

The present work is an attempt to find a simple technique which will give reliable results, and approaches the problem from a somewhat different angle. An attempt is made to find a simple nutrient medium, the ingredients of which can be shown to be initially free from sulphonamide antagonizers. The experimental work is concerned first with the preparation of a suitable medium and standardization of the technical details of the test, and secondly with an attempt to correlate laboratory and clinical findings in urinary infections, for which sulphonamides are still frequently used.

Experimental

Determination of Sulphonamide-antagonizer Content and Choice of Medium.—The test organism was a strain of *Escherichia coli*, sensitive to 0.4 mg.% of sulphathiazole as estimated by the tube method using absorbed broth (Harper and Cawston, 1945). A synthetic agar medium was prepared containing the sulphonamides in a final concentration of 1 mg.%. The sulphonamides used were sulphathiazole, sulphadiazine, sulphamerazine (the components of "sulphatriad"), and sulphamezathine. The surface of each plate was diffusely inoculated with the test organism. Heatley cups were placed on the surface and each received 5 drops, from a standard dropper, of a 10% solution of different commercial brands of peptone : the plates were incubated at 37° C. Because of the high concentration of sulphonamide, growth did not occur except in the vicinity of those cups which contained sulphonamide antagonizer (Figs. 1a and 1b). The area of growth around each cup was a measure of the amount of antagonizer present. Woods (1940) showed that *p*-aminobenzoic acid (P.A.B.) had the same effect as the antagonistic substances in peptones, and we therefore measured the antagonizer content in terms of "P.A.B. equivalents" by performing control experiments with varying concentrations of P.A.B. (Table I). It is interesting to note that, whereas with sulphathiazole, sulphadiazine, and sulphamerazine the zones of growth were similar in size, with sulphamezathine the zones were larger. This was probably due to the lower specific activity of sulphamezathine. With all four drugs, however, Evans peptone had the lowest antagonizer content ; eight batches were tested with consistent results. Lablemco, tested in the same way, showed little or no antagonizer activity with sulphathiazole and sulphadiazine, although there was a definite inhibitory effect against sulphamerazine and sulphamezathine (Table I).

In view of these findings a base medium was chosen, containing minimal amounts of antagonizer, and consisted of 0.5% Evans peptone, 0.3% Lab-lemco, 1.5%

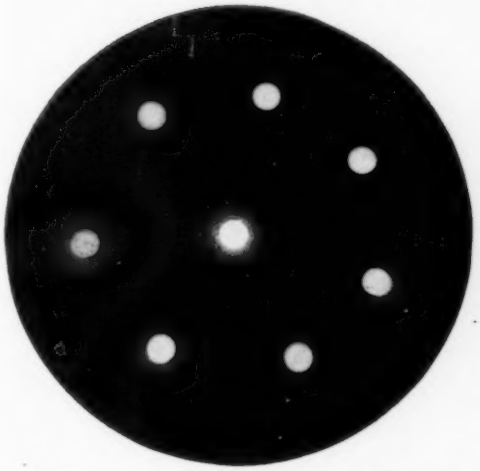


FIG. 1a.—The petri dish contained a synthetic agar medium with 1 mg.% sulphadiazine. Different brands of peptones were placed in the Heatley cups round the periphery. Haloes of bacterial growth surround all but two, which contained Evans and Oxoid peptones respectively, and are due to diffusion of antagonizer. The halo round the central cup, which contained Lab-lemco, is not produced by bacterial growth but by diffusion of pigments.

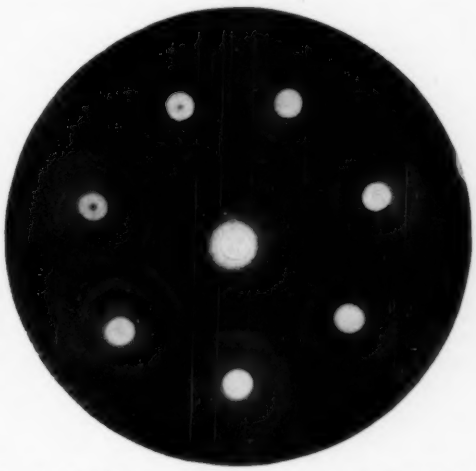


FIG. 1b.—The plate was prepared in a similar way to that in Fig. 1a except that the medium contained 1 mg.% sulphamezathine instead of sulphadiazine. The haloes of bacterial growth are generally larger; the effects round Evans and Oxoid peptones remain slight. There is a small halo round the cup containing Lab-lemco.

TABLE I

CONTENT OF SULPHONAMIDE ANTAGONIZER IN VARIOUS COMMERCIAL PRODUCTS

0·5% Solution of	Sulphonamide Antagonizer Expressed as "PAB" Equivalent (mg.%)			
	Sulphathiazole	Sulphadiazine	Sulphamerazine	Sulphamezathine
Bacto-tryptone	0·025	0·025	0·05	0·05
Neopeptone	0·025	0 05	0·05	0·05
Difco proteose peptone No. 3	0·025	0·025	0·025	0·05
Difco proteose peptone No. 2	0·05	0·05	0 05	0·05
Difco proteose-peptone ..	0·025	0·005	0·005	0·05
Oxoid peptone	0·005	Nil	0·005	0·005
Evans peptone	0·001	Nil	Nil	0·0025
Lab-lemco (0·3%)	0·0006	Nil	0·003	0·002

agar (N.Z.), and 0.5% sodium chloride. When sensitive strains of *E. coli* were tested on this medium by the usual disc method of antibiotic testing appreciable zones of inhibition were obtained, but the zones, on closer scrutiny, contained numerous minute colonies. These were thought to be due to the presence of the traces of antagonizer in the medium, and laked horse blood was added in varying concentration to overcome this effect. It was found that as little as 0.25% blood in the base medium was sufficient to clear the zones of the minute colonies. The medium finally chosen for the investigation, therefore, had the following composition :

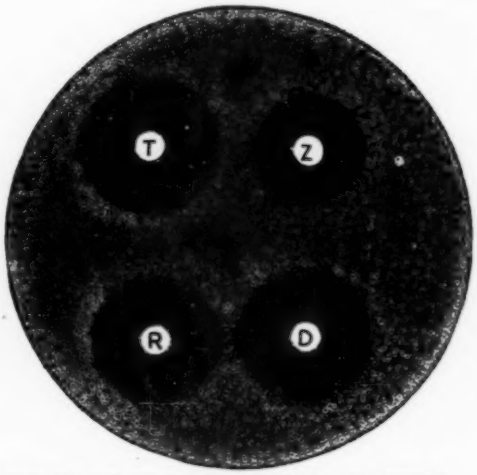
Evans peptone	5.0 g.
Lab-lemco	3.0 g.
Agar powder (N.Z.)	15.0 g.
Distilled water	1 litre

(With the omission of NaCl whole horse blood could be added directly to the medium with spontaneous laking.) The ingredients were dissolved in the distilled water, clarified by filtration through paper pulp after adjusting to *p*H 7.2–7.4 and sterilized by autoclaving for 20 minutes at 15 lb. pressure per sq. in. The medium was bottled in 100 ml. quantities. Before use it was melted, cooled to 50–56° C., and 0.5% of oxalated horse blood added. After thorough mixing plates were poured and stored in the refrigerator for at least 12 hours before use to allow complete neutralization of antagonizer.

Preparation of Sulphonamide-impregnated Blotting Paper Discs.—The organisms were tested against sulphathiazole, sulphadiazine, sulphamerazine, and sulphamezathine. The sodium salts, dissolved in distilled water, were tested in concentrations of 200, 300, and 400 mg. per 100 ml. After a large number of trials 300 mg. per 100 ml. was found to give the best correlation with clinical results and this concentration was therefore adopted for the test.

An office punch was used to cut out discs, 6 mm. in diameter, from Ford blotting paper (428 Mill), a different colour being used for each sulphonamide. The discs were placed in 1 oz. universal containers and 0.8 ml. of sulphonamide solution added for every 100 discs. The containers were then sterilized by autoclaving at 10 lb. per sq. in. for 20 minutes. This method was found to give results sufficiently reproducible for routine use.

Inoculum Size.—It is known that the bacteriostatic activity of sulphonamides is related to the inoculum

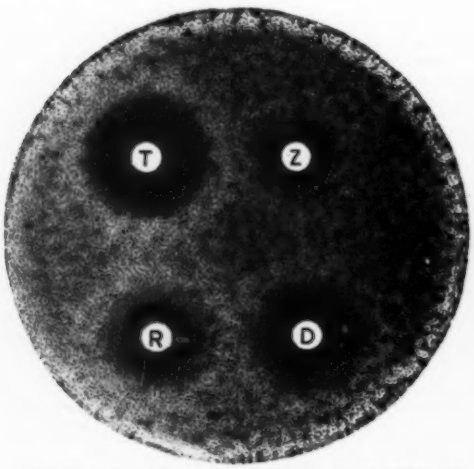


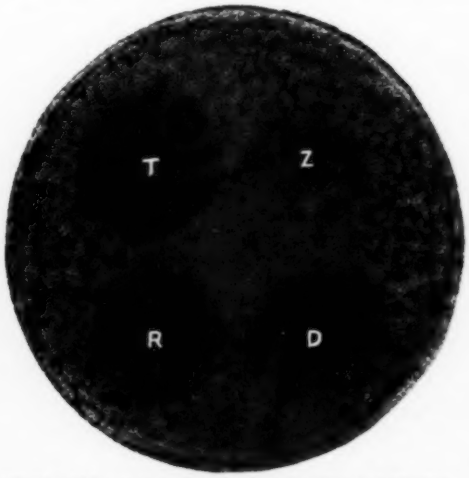
FIGS. 2*a* and *b*.—The plates shown were inoculated with the same strain of *E. coli*, and discs containing the four sulphonamides were placed on the surface of each. In Fig. 2*a* the plate contained base medium plus 0·5% laked horse blood: five hours' diffusion was allowed, the discs were removed, the plate inoculated and incubated overnight. Clear zones of inhibition are seen except round the disc containing sulphamezathine. The plate in Fig. 2*b* was prepared in the same way, but there was no prediffusion, the discs being applied immediately after inoculation. Under these conditions sulphamezathine has produced a clear zone of inhibition similar to those of the other three drugs.

T = Sulphathiazole D = Sulphadiazine R = Sulphamerazine Z = Sulphamezathine

size (Fleming, 1940; Colebrook and Francis, 1941). Like Kokko (1947) and Evans (1948) we found that the most satisfactory results were obtained with inocula which gave just discrete colonies in the uninhibited zone. This was readily obtained by lightly touching a colony with the point of a wire and emulsifying it in about 3 ml. of sterile saline.

Prediffusion.—Earlier workers (Kokko, 1947; Evans, 1948) found that the best results were obtained if the sulphonamide discs were placed on the surface of the plate five hours before inoculating. This method of prediffusion was therefore used in the first series of tests. While most organisms showed good zones of inhibition with the components of sulpha-





FIGS. 3*a* and *b*.—The medium was absorbed with 6% laked horse blood, which was removed by heat precipitation and filtration. In Fig. 3*a* the sulphonamides were allowed to diffuse into the agar for five hours before the plate was inoculated: in Fig. 3*b* the sulphonamide discs were placed on the surface of the medium after inoculation. In both cases the zones of inhibition are smaller than in Fig. 2*b* and the organisms appear "resistant" to sulphamezathine.

triad, almost half the organisms appeared insensitive or only slightly sensitive to sulphamezathine (Fig. 2a). Some of the patients from whom these organisms were obtained were, however, successfully treated with a course of sulphamezathine, suggesting false results *in vitro*. At first it was supposed that insufficient blood had been added to remove all the sulphonamide antagonizer, and we therefore retested the effect of sulphamezathine incorporating increasing concentrations of laked horse blood in the base medium. However, as the concentration of blood increased so the organisms apparently became still less sensitive to the drug, suggesting that laked horse blood itself contained a sulphamezathine inhibitor: to prove this the organisms were tested on the base medium alone and considerably larger zones of inhibition were obtained. The time of contact between the sulphamezathine and the blood was then reduced to a minimum by inoculating the plate before the sulphamezathine discs were placed on the surface (that is, without prediffusion), and again good zones of inhibition were obtained (Fig. 2b). For this reason 0.5% blood was retained in the final medium to eliminate fine growth within the zones of inhibition, but the plates were inoculated without prediffusion of the sulphonamide to minimize the inhibitory effect of the blood on sulphamezathine.

Comparison with a Medium Absorbed with 6% Laked Horse Blood.—The finding of this apparent inhibitory effect of blood on sulphamezathine led us to compare results on the selected medium with those obtained on a medium containing high concentrations of blood. The method chosen for comparison was that of Evans (1948) which is a well-known blotting paper disc method.

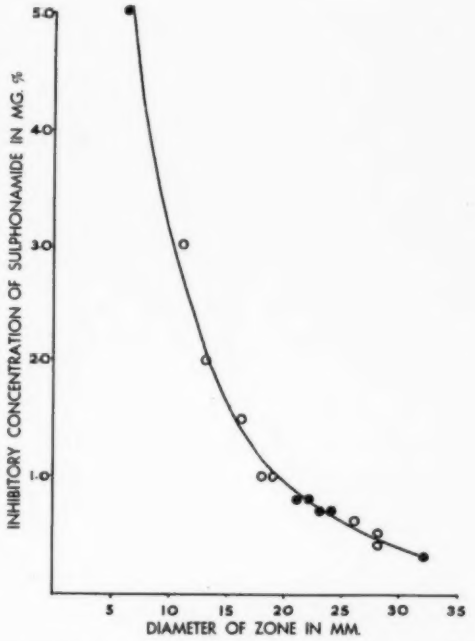
Evans's medium was prepared by incubating sterile nutrient broth (containing oxoid peptone) with 6% laked horse blood for 24 hours, steaming for half an hour to precipitate the blood, filtering through paper pulp, and solidifying with 1.5% powdered agar.

A sensitivity test was performed in parallel with one on our medium, the sulphonamide-impregnated blotting paper discs being placed on the surface of the plates after inoculation.

Fig. 3a shows that the zones of inhibition obtained on the absorbed-agar medium were considerably smaller than those on our medium (Fig. 2b): in fact the organism appeared completely resistant to sulphamezathine. Even when the sulphonamides were allowed to diffuse into the absorbed-agar medium for five hours before the plate was inoculated the zones were reduced in size (Fig. 3b). Similar results were obtained when the experiment was repeated with a medium prepared from a different brand of peptone.

From these results we concluded that a high concentration of horse blood can inactivate sulphonamide, particularly sulphamezathine. This effect is still present after the blood is precipitated by boiling and removed by filtration.

FIG. 4.—Graph obtained by determining the inhibitory sensitivity of a number of species of *E. coli* to sulphathiazole and sulphamezathine and plotting against the respective zone diameters obtained by the plate test.
●—● Strain tested with sulphathiazole
○—○ Strain tested with sulphamezathine



Correlation of Sensitivity of the Organism with Size of the Zone of Inhibition.—The sensitivities of a number of organisms to the four sulphonamides were determined by the tube test (using a broth containing the same ingredients as the base medium, with 0.5% laked horse blood) and plotted against the diameters of the corresponding zones of inhibition with the plate test. The four curves thus obtained were so similar that for practical purposes they could be considered as one curve (Fig. 4). By measuring the diameter of the zone produced by any one sulphonamide on an unknown organism its approximate inhibitory concentration could be obtained from the curve.

Two out of 39 sensitive organisms showed a fine growth inside the zones of inhibition despite the presence of laked horse blood in the medium. However, their tube sensitivities coincided with those predicted from their zone diameters and it therefore seemed reasonable to treat them in the same way as organisms showing no growth within the inhibition zone. The significance of this observation requires further study.

Method and Interpretation of the Routine Sensitivity Test

The medium was prepared as described, and plates were stored for at least 12 hours before use. They were dried for half an hour at 37° C. and seeded with the standard inoculum by flooding; excess was removed and the plates were placed in the 37° C. incubator until dry (10–15 minutes). A disc of each sulphonamide was then placed on the surface of the medium and the plates were incubated overnight.

Interpretation.—The method of interpretation was chosen after consideration of reported blood levels. Sulphatriad and sulphamezathine in the doses normally used for urinary infections (1 g. six or eight hourly) produce blood levels which are most commonly between 2 and 4 mg. per 100 ml. (M.R.C. War Memo, 1943; Bullowa and Ratish, 1944; Volini, Engbring and Schorsch, 1945) although variations among individuals of from 1 to 23 mg.% have been reported (Clark, Murphy, and Flippin, 1943). Organisms with zones of inhibition of more than 15 mm. and therefore sensitive to less than 1.5 mg.% were reported as "sensitive": organisms with zones between 10 and 15 mm. and therefore sensitive to from 1.5 to 3.0 mg.% as "slightly sensitive," and those with zones of less than 10 mm. as "resistant."

Comparison of Laboratory and Clinical Findings

In-vitro Findings.—Sixty-three organisms from 50 patients with urinary infections were isolated and tested. Forty-three were *E. coli*, four *A. aerogenes*, four *P. vulgaris*, and two atypical coliform bacilli and 10 *Strep. faecalis*. Nineteen organisms were completely resistant to the four sulphonamides: Table II shows the species distribution.

TABLE II
SULPHONAMIDE SENSITIVITY OF THE DIFFERENT SPECIES OF ORGANISMS

Organism	No.	Sensitive	Resistant
E. coli	40	37	3
A. aerogenes	4	1	3
P. vulgaris	6	4	2
Atypical coliform	3	2	1
Strep. faecalis	10	0	10
Total ..	63	44	19

With the exception of *Strep. faecalis*, these results cannot be accepted as the natural sensitivities of the organisms, as all the resistant Gram-negative bacilli were isolated from patients who had been treated with sulphonamide some time previously. Two of these resistant organisms were found to be insensitive to 60 mg.% of sulphathiazole.

Clinical Findings.—Fifty patients with urinary infections were studied: as far as possible they were consecutive cases and treatment was independent of the laboratory results. A specimen of urine was obtained in each case before treatment and the presence of pus and a heavy growth of organisms were accepted as proof of urinary infection. The patients in this series were given either sulphatriad or sulphamezathine, 1 g. six or eight hourly for five to seven days (in some cases potassium citrate was also given). A second specimen was examined in most cases 24 to 48 hours after the drug had been discontinued: if organisms were present their sensitivity was retested. Sixteen of the 50 patients suffered from acute pyelonephritis, 25 had infections following operations (mainly gynaecological), and nine patients had acute episodes in chronic urinary disease. In 30 patients the infection had been present from one to seven days, in 20 symptoms had been present for over 10 days. From the 50 patients 63 organisms were isolated and incriminated as the cause of infection. In 10 patients a mixed infection of *Strep. faecalis* and a Gram-negative bacillus was present, and in three cases two species of Gram-negative bacilli were isolated. The correlation between the results *in vitro* and the clinical findings is shown in Table III. All but one of the 39 patients with

TABLE III
CORRELATION BETWEEN SULPHONAMIDE SENSITIVITY TEST AND CLINICAL RESULTS

Drug	Laboratory Report	Total No. of Organisms	Correlation with Clinical Results	
			Yes	No
Sulphamezathine ..	Sensitive	20	20	0
	Slightly sensitive	5	3	2
	Resistant	9	9	0
Sulphatriad ..	Sensitive	19	18	1
	Slightly sensitive	0	0	0
	Resistant	10	9	1
Total		63	59	4

organisms reported as "sensitive" were cured with one course of sulphonamide, even though in 11 of these the infection had been present for more than two weeks: three of these patients had been given a previous course of potassium citrate and two patients chloramphenicol without effect. In one case the organism (*P. vulgaris*) was present after the course of treatment had been completed, although it was found to be still sensitive. A further course of sulphatriad was successful.

The organisms isolated from five patients treated with sulphamezathine were reported as "slightly sensitive." In two of these the infection was overcome with a single course of the drug. In one of

the remaining cases a second course of sulphamezathine was needed before the urine was successfully sterilized. In the other two cases the organisms persisted although one patient received 20 and the other 28 g. of sulphamezathine: second courses of sulphamezathine were not given. One of these last two patients had a post-operative urinary infection and was repeatedly catheterized; the other had acute pyelonephritis and the *E. coli* isolated after treatment was found to be resistant to sulphamezathine. Failure of treatment in these cases was probably due to the narrow margin between the sensitivity of the organisms and the blood levels achieved by the usual dosage of the drug.

Nineteen organisms were reported as "resistant"; 10 of these were *Strep. faecalis* and the rest various Gram-negative bacilli. In 18 of the cases treatment had no effect on the course of the infection and the organisms isolated after treatment were again found to be completely resistant by the plate test. In the nineteenth case, infected with a "resistant" organism, the one specimen received after treatment was sterile, but the infection recurred soon after: this patient had a suprapubic cystotomy with an indwelling catheter. As the organism was resistant to 30 mg.% sulphathiazole, it is difficult to believe that the drug brought about the temporary cure.

Discussion

The method described has overcome some of the difficulties encountered in sulphonamide sensitivity testing. The first of these is the presence of sulphonamide antagonizing substances in the ingredients of nutrient media. These can best be dealt with by using media which, by a relatively simple method, can be shown to contain minimal amounts of sulphonamide antagonizer. A very small quantity of laked horse blood is sufficient to remove this trace of antagonizer. It was, however, shown that with some organisms even small quantities of blood are able to inhibit the action of sulphamezathine. This effect can be minimized by placing the sulphonamide-impregnated discs on the surface of the medium after the plate has been seeded with the test organism.

It was also demonstrated that if large concentrations of blood are used to remove antagonizer there is considerable inactivation of all four sulphonamides, shown by a decrease in the size of the zones of inhibition; this occurs even if the blood is removed by heat precipitation and filtration, and whether the sulphonamide is allowed to prediffuse into the medium or not.

Another problem was the occasional occurrence of organisms which constantly gave a fine growth within the zones of inhibition, even on media containing laked horse blood. It was found that their sensitivities by the tube method corresponded with the diameters of the zones and they therefore were reported as "sensitive"; this appears justified by clinical results.

The final difficulty which is inherent in all plate sensitivity tests was that of selecting the concentration of drug for the test plate so that the results *in vitro* reflect as closely as possible those obtainable *in vivo*. Although no attempt was made to estimate chemically the amounts of sulphonamide in the agar, a simple test was performed to relate the tube sensitivities of the organisms with their zones of inhibition (Fig. 3). In this way it was shown that, using 300 mg.% of sulphonamide in the disc, the concentrations in the agar at the time the organisms were inhibited agreed well with reported blood levels (2–4 mg.%): any organism sensitive to more than 3 mg.% showed no zone or only slight thinning.

In general, organisms appear either very sensitive to the sulphonamides or completely resistant. Owing to the lesser activity of sulphamezathine, organisms occasionally appear only "slightly sensitive" to this drug and if the usual doses are used for treatment the infection may not be cured. A report "slightly sensitive" to sulphamezathine therefore implies that higher doses should be given or that "sulphatriad" should be used instead.

In the present work we have strictly limited ourselves to organisms obtained from urinary infections. It may be that in other circumstances *in vitro* results will not reflect the behaviour of the drug in the body and further work is required before the value of the test can be assessed in other types of infection.

Summary

A simple plate method is described for testing the sensitivities of organisms isolated from urinary tract infections to sulphathiazole, sulphadiazine, sulphamerazine, and sulphamezathine.

The method is based on the choice of a nutrient medium containing minimal traces of sulphonamide antagonizer. The trace of antagonizer is removed by the addition of very low concentrations of laked horse blood, but even the small amounts have inhibitory properties against sulphamezathine, which, however, become marked only if the sulphonamide has been allowed to prediffuse through the medium.

When large quantities of horse blood are added to remove antagonizer the action of all four sulphonamides is interfered with. Because of this false results are likely to be obtained with some of the methods in current use.

In a series of 50 patients with urinary infections the laboratory tests here described showed excellent agreement with subsequent clinical results.

We are extremely grateful to Dr. S. D. Elek for his continued advice and encouragement throughout the course of this investigation. We also wish to thank the Endowment Fund of St. George's Hospital for a grant towards the expenses of the work.

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ANTIBIOTIC SENSITIVITY OF THE GENUS *PROTEUS*

BY

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The classification of the genus *Proteus* is still to some extent controversial, although recent investigations have clarified the position considerably. The basis of the modern work was the demonstration by Moltke (1927) that 194 *Proteus* strains all rapidly hydrolysed urea. For the purposes of this paper, the genus *Proteus* includes all motile, Gram-negative, straight rods which do not ferment lactose, do ferment glucose, and hydrolyse urea rapidly. (With a stab culture in a solid urea-containing medium a positive result is obtained in less than four hours.) On such a basis, Rustigian and Stuart (1943) proposed to include in the genus four species: *P. vulgaris*, *P. mirabilis*, *P. morgani*,* *and P. rettgeri*, which can be readily differentiated on the basis of a few simple biochemical reactions. Cook (1948) studied the cultural characteristics of the genus more fully and showed the relative frequency of the four species.

For the purposes of routine identification it is justifiable to determine the species on the basis of three characteristics only; sucrose fermentation in 24 hours, mannitol fermentation, and indole production, as shown in Table I.

TABLE I

FERMENTATION REACTIONS AFTER 24 HOURS' INCUBATION

	P. vulgaris	*P. mirabilis*	*P. morgani*	*P. rettgeri*
Sucrose ..	AG	−	−	−
Mannitol	−	−	−	A
Indole ..	+	−	+	+

+ = Urease. − = Lactose. AG or A = Glucose.

P. rettgeri has had a rather chequered career. It was originally described by Rettger and Harvey in 1908 as the cause of a disease in fowls, and has been known at different times as *Bacillus rettgeri*, *Bacterium rettgeri*, *Eberthella rettgeri*, and *Shigella rettgeri*. Its inclusion in the genus *Proteus* is based mainly on its similarity to the other species in respect of urease production, but it has other features in common. On 1% agar it shows the characteristic swarming behaviour which *P. vulgaris* and *P. mirabilis* give on most solid, non-inhibitory media. *P. rettgeri* is a common and not unimportant organism. Ten per cent. of faecal *Proteus* strains isolated by Cook (1948) were *P. rettgeri* and an even larger proportion is found among strains from urinary infections.

* This organism is usually referred to as *P. morganii* in the American literature. I have followed the usual English spelling, which seems to have historical priority.

Material and Methods

Forty-six *Proteus* strains have been tested for sensitivity to streptomycin, chloramphenicol, aureomycin, and terramycin. These comprise all strains isolated in the Oxford Public Health Laboratory and in the Bacteriology Department of the Radcliffe Infirmary, Oxford, during October and November, 1952. None have been omitted except that only one strain from each patient has been included. The majority were from hospital in-patients, but many were from faecal specimens sent in from further afield, so that these should be a reasonably representative group.

The usual tube technique has been followed in estimating sensitivity. Doubling dilutions of a solution of the antibiotic were mixed with a weak suspension of organisms from a young culture in Wright's broth and incubated overnight. All strains were tested over a range from 50 units or microgrammes per millilitre down to 1½ units or microgrammes per ml. No attempt has been made to investigate the sensitivity of these organisms to higher concentrations, since sensitivity to higher concentrations is of little therapeutic value.

Results

The sensitivities of 46 *Proteus* strains grouped according to species are shown in Tables II, III, IV, and V. In Tables II to V a dash indicates resistance to 50 units or μg. per ml., and the numbers the lowest concentration to which the organism was sensitive.

Twenty strains of *P. mirabilis* were all reasonably sensitive to chloramphenicol. Streptomycin seems

TABLE II
RESULTS OF SENSITIVITY TESTS ON 20 STRAINS OF *PROTEUS MIRABILIS*

Streptomycin	Chloramphenicol	Aureomycin	Terramycin
12	12	—	—
6	6	50	—
6	12	50	—
12	1·5	50	—
12	6	—	—
12	12	—	—
12	12	—	—
12	12	—	—
12	6	—	—
12	3	—	—
25	25	—	—
25	12	—	—
50	25	—	50
50	1·5	—	—
50	12	—	—
50	12	—	—
25	3	—	50
—	3	—	—
12	12	—	—
6	12	—	—

TABLE III
RESULTS OF SENSITIVITY TESTS ON EIGHT STRAINS OF *PROTEUS RETTGERI*

Streptomycin	Chloramphenicol	Aureomycin	Terramycin
—	—	3	25
—	—	3	1·5
—	—	3	6
—	—	12	1·5
—	—	12	3
—	—	3	3
—	—	12	3
—	—	1·5	25

TABLE IV
RESULTS OF SENSITIVITY TESTS ON 12 STRAINS OF *PROTEUS MORGANI*

Streptomycin	Chloramphenicol	Aureomycin	Terramycin
—	6	6	—
—	1·5	25	—
1·5	3	3	50
6	3	12	1·5
6	12	25	3
—	50	50	—
6	3	12	3
50	—	50	—
6	6	3	1·5
3	6	6	1·5
3	25	—	—
—	3	25	3

TABLE V
RESULTS OF SENSITIVITY TESTS ON SIX STRAINS OF *PROTEUS VULGARIS*

Streptomycin	Chloramphenicol	Aureomycin	Terramycin
12	6	25	12
12	6	6	12
—	—	25	3
—	—	3	3
—	6	—	—
—	3	—	—

to be slightly less effective, but the only strain resistant to 50 units per ml. was from a patient under treatment with streptomycin for pulmonary tuberculosis. On the other hand, all of these organisms were relatively resistant to aureomycin and terramycin.

The results with eight strains of *P. rettgeri* are in complete contrast. All strains were resistant to streptomycin and chloramphenicol, but all were sensitive to aureomycin and terramycin.

Twelve strains of *P. morgani* gave less consistent results, but on the whole this seems to be the most sensitive group. Most of them seem to be sensitive both to chloramphenicol and to aureomycin, unlike the *mirabilis* and *rettgeri* types.

Only six *P. vulgaris* strains are included in this series and these showed greater variation in the pattern of antibiotic sensitivity than the other species. However, it may be relevant to record the fact that these six can be divided into three groups of two strains each, giving three pairs of strains in each of which similar fermentation reactions are apparently correlated with similar antibiotic sensitivities. First, two strains which fermented sucrose, maltose, and salicin were sensitive to all four antibiotics. Secondly, two which fermented sucrose and maltose but not salicin were sensitive to aureomycin and terramycin but not to streptomycin or chloramphenicol. The third group comprises two strains which fermented sucrose but not maltose or salicin and were sensitive to chloramphenicol only. Evidently this is the most heterogeneous group both in its biochemical reactions and in its antibiotic sensitivity pattern.

Discussion

The pattern of sensitivity to antibiotics of *Proteus* strains seems to be correlated to some extent with their classification into four species. Two of the species show quite striking uniformity in that *P. mirabilis* was sensitive to streptomycin and chloramphenicol and *P. rettgeri* was sensitive to aureomycin and terramycin. However, it must be emphasized that this is a comparatively small series collected in one centre over a short period of time, and until a much larger number of observations have been accumulated it would be unwise to infer that the findings in this series are universally applicable.

It is suggested that the sensitivities of *Proteus* strains should be tested with streptomycin, chloramphenicol, aureomycin, and terramycin as, with four exceptions, the strains were sensitive to 12 units or microgrammes per ml. or less of at least one antibiotic.

Summary

The sensitivity of 46 *Proteus* strains to streptomycin, chloramphenicol, aureomycin, and terramycin has been investigated over a range of 1.5 to 50 units or microgrammes per millilitre.

Twenty strains of *P. mirabilis* were sensitive to chloramphenicol and all but one to streptomycin, but were relatively resistant to aureomycin and terramycin.

Eight strains of *P. rettgeri* were sensitive to aureomycin and to terramycin but not to streptomycin or to chloramphenicol.

Twelve strains of *P. morgani* were on the whole more sensitive than those of the other species, most of them being sensitive both to chloramphenicol and to aureomycin in contrast to *P. mirabilis* and *P. rettgeri*.

In the *P. vulgaris* group, greater variation was found.

My thanks are due to Dr. R. L. Vollum and Dr. G. T. Cook for constant advice and encouragement.

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THREE CASES ILLUSTRATING THE PRESENCE OF ARGENTAFFIN (KULTSCHITZKY) CELLS IN THE HUMAN GALL-BLADDER

BY

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Occasionally typical argentaffin (Kultschitzky) cell or so-called "carcinoid" tumours have been described in the human gall-bladder (Joël, 1929; Porter and Whelan, 1939; Bosse, 1943), although I have found no mention in textbooks of histology or pathology of the presence of such cells in the mucosa. There is, however, one case report (Kerr and Lendrum, 1936) of a papilloma of this organ covered with intestinal epithelium containing both Paneth and argentaffin cells. I propose to describe three cases in which the latter type was present.

Material and Methods

The material was obtained from operation cases in which extra sections were cut from routine formalin-fixed paraffin blocks and stained by an alkaline silver method, either that of Masson and Hamperl as described by Jacobson (1939) or Gomori's (1948) hexamine-silver nitrate technique. In Case 3 the argentaffin cells were numerous and several histochemical stains were applied.

Being surgical material, most of the gall-bladders examined showed evidence of chronic inflammation. Nicholson (1923) has already given a masterly description of the pathological changes in chronic inflammation and, in particular, has drawn attention to areas of mucous gland formation, a phenomenon which he has referred to as indirect metaplasia. He found such areas in 24 out of 36 inflamed organs removed at operation and remarked that the percentage would probably have been increased had more sections been examined. He mentioned that Aschoff (1905), Schridde (1909), and Bodnár (1922) had previously drawn attention to the presence of mucous glands upon the surface of the fundus of the inflamed gall-bladder. Schridde (1909) also demonstrated the origin of these glands from the epithelium of Luschka's tubes in a case of cholecystoduodenal fistula.

Nicholson demonstrated how these tubular mucous glands change their type in a high proportion of cases and assume all the structural characters of gastric glands identical with those of the pylorus or those of Brunner. His Fig. 6 confirms this finding and presents a very similar picture to that seen in numerous areas in each of the three cases described below. Seventeen of Nicholson's series of 24 gall-bladders which contained mucous glands possessed those of the gastric type as well in varying numbers and in all stages of differentiation. He analysed the changes that eventually end in the formation of such gastric glands in the gall-bladder and considered that they were of two kinds, namely, regressive, leading to the production of tubular mucous glands and other changes characteristic of the intestines, and progressive, culminating in the production of typical glands of the gastric type. (He considered Brunner's glands to be of the gastric type.)

As Nicholson has given such a full description of the changes in the gall-bladder epithelium in chronic inflammatory lesions, it only remains for me to demonstrate that argentaffin cells also occur both in the high columnar epithelium covering the surface of the mucous membrane and in the glandular acini. Morphologically the cells are almost always conical with a broad base in contact with the basement membrane and tapering towards a narrow elongated apex, which usually falls short of the lumen of the gland or the free mucosal surface as the case may be. The nucleus is usually situated near the middle of the cell and is vesicular with chromatin aggregations often more or less evenly distributed beneath the nuclear membrane (Fig. 3): such a nuclear pattern has been beautifully depicted by Ciaccio (1906). The intracellular granules lie between the nucleus and the basement membrane, that is, they are infranuclear. The histological appearances of these

cells are, in fact, identical with those of the intestinal argentaffin cells which have been fully described by other workers (Cordier, 1926 ; Masson, 1928 ; Gillman, 1942).

Case Reports

Case 1.—The wall of the gall-bladder, that of a man aged 71, is up to 0.3 cm. thick and its mucosa in some places is thrown into irregular papillary processes and in others shows both Luschka's tubes penetrating into the muscularis and foci of glandular acini, the latter arising both from the surface epithelium and from the tubes.

Scattered very irregularly amongst the high columnar cells covering the papillary processes are cells with alkaline silver-reducing infranuclear granules which morphologically resemble intestinal argentaffin cells (Fig. 1). Occasionally such cells are present in the glandular acini, a feature also depicted in Fig. 1. Goblet cells are rarely seen on the surface epithelium, and, when present, bear no relation to the argentaffin cells : both apparently are scattered indiscriminately and very irregularly amongst the high columnar cells. Paneth cells are not present. The underlying wall is thickened and its musculature is partly replaced by fibro-cellular connective tissue. The submucosa is only sparsely peppered with chronic inflammatory cells ; in fact, the original chronic infective process shows no histological evidence of activity.

Case 2.—The gall-bladder, that of a woman aged 60, measures 9.5 cm. in length and up to 2.8 cm. in diameter, with a wall averaging 0.2 cm. in thickness. Its mucosa has a wrinkled surface.

Microscopically there is a considerable overgrowth of fibrous tissue involving all three coats. Approximately 5.0 cm. of mucosal surface is included in the sections, although, in parts, the covering epithelium is denuded. Luschka's tubes penetrate deeply into the mucosa, especially in one area where there are

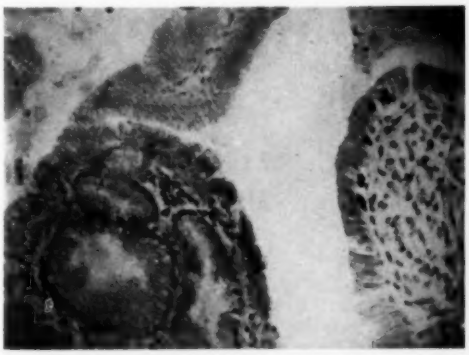

FIG. 1.—Case 1, showing argentaffin cells, both on a papillary projection of the mucosa (right side) and a solitary cell in a glandular acinus (lower left corner). Masson-Hamperl's ammoniacal silver stain. × 200.

FIG. 2.—Case 2, showing argentaffin cells, both solitary and in groups, in glandular acini. One group (in centre) has been sectioned obliquely. Masson-Hamperl's ammoniacal silver stain. × 200.

numerous coiled glands, many being of Nicholson's " gastric " type. Here and there in this nidus of acini are cells morphologically resembling the argentaffin cells of the intestine and containing infranuclear ammoniacal silver-reducing granules (Fig. 2). In one area, in adjoining acini (almost certainly part of the same coiled gland system), there are two clumps of such cells, the larger containing eight cells in apposition. In addition, there is an occasional isolated argentaffin cell in the same gland system (Fig. 2). When present in mucous glands these cells are, as in Brunner's glands, usually found lying at the periphery of the acinus against the basement membrane and separated from the lumen by mucous cells. In the papilliferous mucosa overlying this group of glands there are goblet cells and an occasional argentaffin cell amidst the high columnar epithelial cells. Elsewhere in the mucosa there are numerous small foci of glandular acini which, however, contain no argentaffin cells. Paneth cells are absent throughout the mucosa.

Case 3.—The specimen is that from a woman aged 49. The macroscopic description of this specimen is not available. Microscopically the wall is slightly thickened due to a moderate overgrowth of fibrous tissue involving the submucosa, muscularis, and subserosa. The covering mucosa is papilliferous in places and partly denuded of its overlying epithelium. The most prominent feature throughout most of the mucosa is a large number of tubular glands which

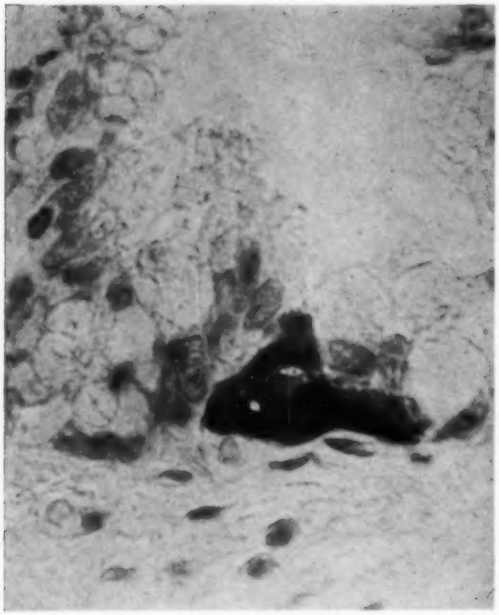

FIG. 3.—Case 3, showing a clump of argentaffin cells in a glandular acinus. In the cell on the extreme right of the clump, the typical chromatin pattern in the nucleus can be discerned. Gomori's hexamine-silver nitrate stain. × 900.

have arisen, both from the surface epithelium and from deeply penetrating Luschka's tubes, the latter having penetrated almost completely through the muscularis in some places. The glands are of both the types described by Nicholson (1923), but the majority belong to his " gastric " variety and do, in fact, closely resemble Brunner's glands. The mucus in both types of glands gives a strongly positive reaction with the periodic-acid-Schiff stain for demonstrating the 1,2-glycol linkage (as would occur in mucopolysaccharides). Argentaffin cells are very unevenly distributed both amongst the glandular mucous cells and the tall columnar surface epithelium. Quite often they appear in clumps (Fig. 3), and in one relatively small group of glands, approximately 1 mm. in diameter and just beneath the surface epithelium, 32 such cells are present, one clump alone containing 13 in apposition. In other areas numerous glandular acini are seen without a single argentaffin cell. No Paneth cells are present. The lamina propria is peppered with chronic inflammatory cells, mainly lymphocytes and plasma cells, and there is also capillary congestion of a moderate degree.

The cells under discussion react positively with the following stains, as in the case of the intestinal argentaffin cells : (1) Gomori's (1948) hexamine-silver nitrate method ; (2) Cordier and Lison's (1930) diazo dye method : the technique as described by Jacobson (1939) was used and the particular dye was " echtrotsalz " supplied by G. T. Gurr, London ; (3) Schmorl's ferric ferricyanide reduction test (Lillie and Burtner, 1953) : the granules stain a blue-green colour.

As only formalin-fixed, paraffin-embedded blocks are available, I have been unable to carry out Baker's acid haematein and pyridine extraction test for phospholipines. It has recently been shown (Christie, 1954) that the granules give a positive reaction in that they stain black in the first part of the test but not in the second—namely, the pyridine extraction-acid haematein test.

The chromaffin reaction could not be observed as it cannot be applied to tissues previously treated with formalin (Pearse, 1953).

Discussion

It is of paramount importance to decide first whether the cells under discussion are, in fact, argentaffin (Kultschitzky) cells such as occur in the intestine. That this is so can hardly be in doubt both from morphological appearances and staining reactions. Masson (1928) has given an admirable account of these cells in the human intestine and his description applies equally well to the cells described in the above three cases. The granules within these cells also show histochemical similarities with the intestinal argentaffin cells, for they give a positive reaction with Cordier and Lison's diazo dye technique, and also possess strongly reducing properties as shown by their ability to reduce both alkaline silver and ferric ferricyanide solutions. Whilst there are other substances occurring in the tissues which have equally strong reducing tendencies, they are not concentrated as fine granules in such a characteristic infranuclear position within the cell as in both these cells and in the typical argentaffin cell of the human intestinal mucosa.

It is very likely that such cells are frequently present in areas of intestinal metaplasia in inflamed gall-bladders but have not been noted, as the usual stains employed in routine histological work do not show them up very clearly, although, if the eosin be properly differentiated in the standard haematoxylin and eosin stain, they can be fairly easily discerned if sought after, for the characteristic granules are quite strongly eosinophilic (Masson, 1928). If the fixative employed contains alcohol then the granules are no longer seen by any staining technique even if formalin is also present in the fixative (Masson, 1928). However, formalin or formalin-containing mixtures are not the only fixatives of these cells as the granules are also preserved by potassium dichromate which gives them a yellow tint (even

in the complete absence of formalin) and by osmium tetroxide which both preserves and blackens them due to its reduction to lower oxides.

Extensive histochemical studies could not be undertaken as only formalin-fixed tissue was available.

A point of considerable interest is the very uneven distribution of these cells. Sometimes, as in Cases 2 and 3 (Figs. 2 and 3, respectively), small clumps of them are seen. In the latter case such clumps contain more cells in apposition with one another than is usually seen in intestinal crypts of Lieberkühn. (I have previously once seen such an aggregation in a small cystic space lined by intestinal epithelium in a teratoma of the ovary.) They do not appear to be related in any specific way to the other cell types seen in metaplastic areas, such as goblet or mucous cells, but rather seem to occur for no apparent reason. In areas of intestinal metaplasia in the stomach, Paneth cells are also often seen, but they have not been observed in the above three cases, although, as mentioned above, Kerr and Lendrum (1936) did see them in their case of a papilloma covered with intestinal epithelium. Although there is some evidence of an active low-grade chronic infective process in the underlying mucosa in the second and third cases, the actual inflammatory lesion, as judged by histological evidence, is no more severe in the mucosa where the argentaffin cells are aggregated than where they are absent.

I have thus no explanation to offer concerning the function of these cells in the sites where they are present, or, for that matter, in the gall-bladder as a whole, except to say that it appears they are likely to occur in intestinal epithelium wherever it may arise. However, their presence in such epithelium is certainly not invariable and this is also the case in areas of intestinal epithelium in teratomata. The presence of these cells in clumps does suggest one or other (or perhaps both) of two possibilities; namely, either that when conditions are such in a particular area for one cell-type to appear, then the stimulus affects the differentiation of more than one stem cell with the consequent production of a group of similar cells; or, alternatively, that when one cell differentiates in a particular direction such as to form an argentaffin cell, then this cell remains true to type for a time at least, and divides to form similar daughter cells which, in turn, may divide to produce a group of specialized cells as in Fig. 3 of Case 3. The presence of such clumps also suggests a likely site for possible tumour formation.

The occurrence of argentaffin cells in this organ, as in teratomata, indicates that direct absorption of precursor substances from the intestine is not esssential for the formation of the chemical substance (or substances) composing the granules.

Summary

Three cases are described in which chronically inflamed gall-bladders removed at operation contain areas of intestinal metaplasia with the presence of typical argentaffin (Kultschitzky) cells both amongst the high-columnar surface epithelial cells and within the mucous glands. The identity of these cells is established both on morphological and histochemical grounds. An explanation is thus afforded for the rare carcinoid tumours of this organ.

I am indebted to Mr. F. E. Speed for the photomicrographs and to Mr. C. G. Chadwin for the histological sections.

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ESTIMATION OF PLASMA PHOSPHATASE BY DETERMINATION OF HYDROLYSED PHENOL WITH AMINO-ANTIPYRINE

BY

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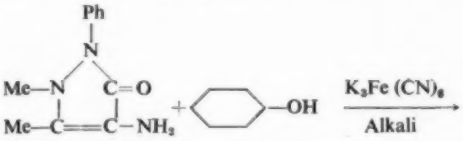
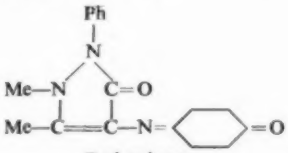
Plasma phosphatase was first determined by Martland (1925) and then by Kay (1930), using glycerophosphate and estimating the inorganic phosphate liberated at 37° C. at a specified *p*H and in a given time. The procedure has been variously modified and simplified by Jenner and Kay (1932), Bodansky (1933), Shinowara, Jones, and Reinhart (1942). Several substrates, other than glycerophosphate, have also been used, e.g., phenyl phosphate (with determination of either the liberated phenol, King and Armstrong, 1934, or phosphate, King, Abul-Fadl, and Walker, 1951), nitro-phenyl phosphate (by the colour of the liberated nitrophenol, Ohmori, 1937; King and Delory, 1939; Bessey, Lowry, and Brock, 1946), naphthyl phosphate (by determination of naphthol by a diazo method, Seligman, Chauncey, Nachlas, Manheimer, and Ravin, 1951), phenolphthalein phosphate (by the colour of alkaline phenolphthalein, Bray and King, 1943; Huggins and Talalay, 1945). Of these procedures, the Bodansky (glycerophosphate) and the King-Armstrong (phenyl phosphate) are perhaps the most widely used.

King and Armstrong (1934) determined free phenol, liberated from phenyl phosphate by the phosphatase, by either the Folin and Ciocalteu (1927) phosphomolybdic reagent or by the diazo reaction, and preferred the former. Both procedures required the precipitation and removal of the plasma proteins subsequent to the hydrolysis of the substrate. In 1951 Grifols Lucas proposed a procedure for determination of the free phenol, which did not require removal of the proteins. This was based on the 4-amino-antipyrine reaction of Gottlieb and Marsh (1946).

In this method the conditions under which enzymic hydrolysis is carried out are the same as in the King-Armstrong method, as modified by King (1951), the amino-antipyrine reagent (A.A.P.) being used to estimate the phenol liberated. 4-Amino-antipyrine reacts with certain phenolic substances in the presence of alkaline oxidizing agents to produce quinonoid substitution products. These give a red colour proportional to the phenol present, and can be determined colorimetrically. Grifols-Lucas found that A.A.P. does not react with plasma proteins, and their removal is unnecessary when phosphatases are estimated.



4-Amino-antipyrine Phenol



Red quinone

We investigated this method in 1951, but delayed publication until the Grifols-Lucas communication should appear in full. As this has not been published so far as we know, and since findings similar to our own are now offered by Powell and Smith (1954), we now present our results. The original procedure has not been successful in our hands, due to interference by proteins with production of the red quinonoid colour, but, by increasing the concentrations of reagents used, we have overcome this difficulty and have developed a method which is not far short of the King-Armstrong method in accuracy, and which does not require precipitation and separation of the plasma proteins, with a consequent simplification and shortening of the estimation.

Investigation of the Grifols-Lucas Procedure

Method.—The method of Grifols-Lucas is as follows:—

Test.—One millilitre M/100 Na_2 phenyl phosphate + 1 ml. Na_2CO_3: $NaHCO_3$ (6:4, *p*H 10) buffer warmed three min. at 37° C.; 0.1 ml. plasma or serum added and mixed; kept at 37° for 15 min.; 1 ml. M/10 Na_2CO_3 added and mixture removed to room temperature; 1 ml. 0.06% 4-amino-antipyrine (A.A.P.) and 1 ml. 0.24% potassium ferricyanide ($K_3Fe(CN)_6$) added and mixed.

Control.—Same as test, except that plasma is added *after* the M/10 Na_2CO_3.

Standard.—Standard phenol solution (0.02 mg. phenol), 1 ml., + 1.1 ml. Na_2CO_3: $NaHCO_3$ buffer + 1 ml. M/10 Na_2CO_3 + 1 ml. 0.06% A.A.P. + 1 ml. 0.24% $K_3Fe(CN)_6$.

The colours develop immediately, and are read in a photoelectric colorimeter with an Ilford 624 green light filter.

Calculation.—

$$\text{Phosphatase (units/100 ml.)} = \frac{\text{Test}^* - \text{Control}^*}{\text{Standard}^*} \times 0{\cdot}02 \times \frac{100}{0{\cdot}1}$$

(* Readings in extinctions. *E*)

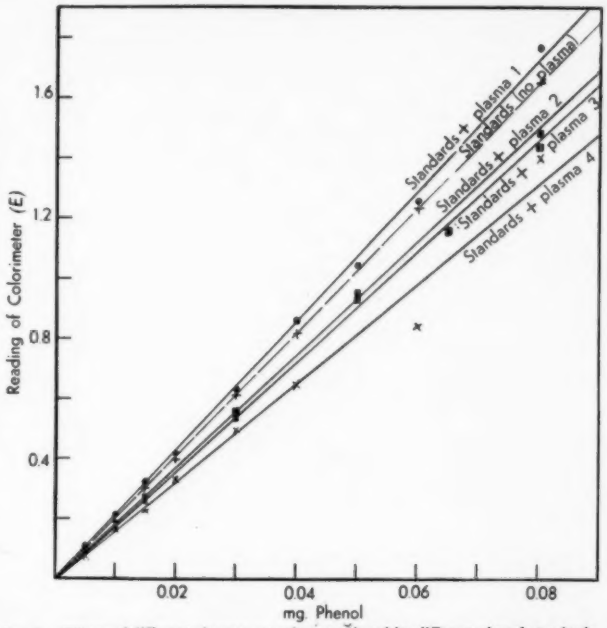


FIG. 1.—Effects of different plasmas on colour produced by different phenol standards.

Comparison of Method with King-Armstrong Procedure.—Twenty-six plasmas (or sera) from hospital routine specimens were analysed by this method in parallel with the King-Armstrong procedure. The results (Table I) were in poor agreement, with a wide scatter, the Grifols-Lucas figures ranging from 60% below the King-Armstrong to 10% above.

TABLE I

COMPARISON OF RESULTS OBTAINED BY KING (1951) AND GRIFOLS-LUCAS METHODS

King Method (K.-A. units)	Grifols-Lucas Method (K.-A. units)	Ratio (K.)/(G.L.)
82·0	83·0	0·99
75·0	72·0	1·04
50·0	49·0	1·02
49·0	51·0	0·97
24·0	22·0	1·09
19·2	13·3	1·44
16·4	13·3	1·23
14·2	11·0	1·29
9·8	8·0	1·22
9·8	3·9	2·5
8·3	8·2	1·01
7·9	6·2	1·27
7·8	7·4	1·05
7·4	5·5	1·35
7·2	5·4	1·34
7·2	3·9	1·85
8·2	5·7	1·44
7·3	6·8	1·08
22·0	18·4	1·2
12·1	9·7	1·25
4·0	1·6	2·5
4·6	4·4	1·04
61·0	38·0	1·6
25·0	21·0	1·19
14·8	11·9	1·25
33·0	36·0	0·91
Average 26·4	19·8	

Effect on Colour of Added Plasma.—With the concentration of reagents used, the presence of plasma has a marked and variable effect on the colour produced in the reaction between phenol and amino-antipyrine (Fig. 1). It is therefore unsound to compare a test containing plasma proteins with a standard which does not. This difficulty was easily overcome by precipitating the plasma proteins (with tungstic acid) and removing them by centrifugation or filtration before adding the Na_2CO_3, A.A.P., and $K_3Fe(CN)_6$. The results then agreed well with the King-Armstrong, but the resulting procedure now had no advantage in simplicity and speed over the latter method.

We therefore sought to modify the method so that the proteins need not be precipitated, and the results nevertheless agree with those by the King-Armstrong procedure and with those for acid phosphatase as described by King (1951).

Modification of the Method

Arrest of Hydrolysis (at End of Incubation Period).—When using the existing Grifols-Lucas method for phosphatase estimations it was noticed that in some cases, particularly those with high phosphatase activity, the colour of both the test and control increased with time, while a standard containing plasma but no substrate gave a steady reading. This was almost

certainly due to continuing phosphatase activity. The addition of M/10 Na_2CO_3 to bring the *p*H to 10.2, favourable for the colour reaction, had not inactivated the enzyme.

By adding a suitable amount of N/2 NaOH the *p*H could be raised, in both alkaline and acid phosphatase estimations, to *p*H 11.5–11.6, thus destroying the enzyme; and by the addition of suitable amounts of M/2 $NaHCO_3$ the *p*H could then be brought back to 10.2 (Table II).

TABLE II

EFFECT OF ADDITIONS OF NaOH (TO DESTROY PHOSPHATASE) AND OF $NaHCO_3$ (TO PRODUCE *p*H 10·2 FOR COLOUR DEVELOPMENT)

	*p*H for Alkaline Phosphatase
A. 1 ml. buffer *p*H 10 + 1 ml. substrate + 0·1 ml. plasma	10·0
B. A + 0·8 ml. 0·5 N NaOH	11·6
C. B + 1·2 ml. 0·5 M $NaHCO_3$	10·2
	*p*H for Acid Phosphatase
D. 1 ml. buffer *p*H 4·9 + 1 ml. substrate + 0·1 ml. plasma	5·0
E. D + 1 ml. 0·5 N NaOH	11·5
F. E + 1 ml. 0·5 M $NaHCO_3$	10·2

Effect on Colour Production in Phenol Standard of Various Strengths of A.A.P. and $K_3Fe(CN)_6$.—Grifols-Lucas's ratio of 1:4 was maintained in each case. Tests were prepared containing either 1 ml. of alkaline buffer *p*H 10, or acid buffer *p*H 4.9, to which were added 1 ml. of 0.01 mg./ml. standard. To the tests with alkaline buffer 0.8 ml. of N/2 NaOH and 1.2 ml. M/2 $NaHCO_3$ were added, and to those with acid buffer 1 ml. N/2 NaOH and 1 ml. M/2 $NaHCO_3$ were added. To all were added 1 ml. of A.A.P. and 1 ml. of $K_3Fe(CN)_6$, the concentrations being varied as shown in Table III. The concentrations 0.6% A.A.P. and 2.4% $K_3Fe(CN)_6$ were chosen as most suitable for the colour reaction, as nearly similar readings were obtained with both alkaline and acid buffer, and a further increase in the strength of reagents appeared to have no advantage.

Calibration Curve Using Phenol Standards of Various Strengths.—Phenol standard, 1 ml., varying between 0.05 mg. and 0.005 mg., was mixed with 1 ml. alkaline buffer or 1 ml. acid buffer. The alkaline buffer standards were treated with 0.8 ml. of N/2 NaOH and 1.2 ml. of M/2 $NaHCO_3$, and the acid with 1 ml. of N/2 NaOH and 1 ml. M/2 $NaHCO_3$. To each was then added 1 ml. of 0.6% A.A.P. and 1 ml. of 2.4% $K_3Fe(CN)_6$. After mixing the solutions the colours were measured using a 624 green Ilford light filter. The colours were first read

TABLE III

EFFECT ON COLOUR PRODUCTION IN A PHENOL STANDARD OF VARIOUS STRENGTHS OF A.A.P. AND $K_3Fe(CN)_6$

Concentration of		Colorimetric Reading ($E \times 100$)	
A.A.P. (%)	$K_3Fe(CN)_6$ (%)	Acid Buffer	Alkaline Buffer
0·06	0·24	11	9·0
0·30	1·2	20	18·5
0·60	2·4	23	22·0
0·90	3·6	25	24·0
1·2	4·8	32	27·0

five min. after adding the reagents, and again after 15 min. No alteration in colour intensity was observed. Fig. 2 shows a straight line in both alkaline and acid buffer, the two lines being nearly superimposed.

Effect of Adding Plasma to Standards in Modified Method.—The effect on colour production of adding 0.1 ml. of plasma to standards was tested. The averages of several determinations made at various times during this investigation are listed in Table IV. The alterations made in the concentrations of alkali, A.A.P., and $K_3Fe(CN)_6$ appear to have so minimized the effect of the plasma proteins on the colour that it can be ignored.

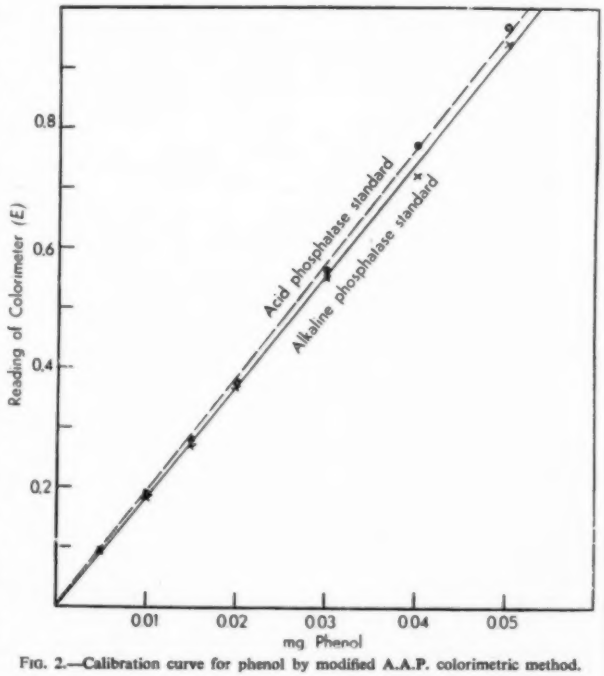


FIG. 2.—Calibration curve for phenol by modified A.A.P. colorimetric method.

TABLE IV

EFFECT OF ADDING PLASMA TO STANDARDS IN THE MODIFIED METHOD

	Standard (mg. phenol)	No.	Alkali Used	Concentration of		Average Reading (E)	
				A.A.P. (%)	$K_3Fe(CN)_6$ (%)	Standard + Plasma	Standard
Alkaline Buffer	0·02	20	1 ml. M/10 Na_2CO_3	0·06	0·24	0·34	0·37
	0·01	20	0·8 ml. N/10 NaOH + 1·2 ml. M/10 $NaHCO_3$	0·6	2·4	0·27	0·23
	0·01	12	0·8 ml. N/2 NaOH + 1·2 ml. M/2 $NaHCO_3$	0·6	2·4	0·225	0·22
Acid Buffer	0·01	20	1 ml. N/2 NaOH + 1 ml. M/2 $NaHCO_3$	0·6	2·4	0·236	0·23

Modified Method Adopted

Solutions.—The following are required :—

pH 10 Buffer.—M/10 sodium carbonate-bicarbonate ($6Na_2CO_3 : 4NaHCO_3$), for which 6.36 g. anhydrous sodium carbonate and 3.36 g. sodium bicarbonate are dissolved in distilled water and made to 1 litre.

pH 4.9 Buffer.—Crystalline citric acid, 21.0 g., is dissolved in water, 188 ml. N-NaOH added, and made to 500 ml. The *p*H should be checked and adjusted to *p*H 4.9 if necessary by dropwise addition of N-NaOH or N-HCl. This solution should be preserved with a few drops of chloroform and kept in the ice-chest.

Substrate.—M/100 disodium phenyl phosphate, 2.18 g., is dissolved in 1 litre of water. The solution should be brought quickly to the boil to destroy any organisms, cooled immediately and preserved with a little chloroform.

Stock Phenol Standard (1 mg. per ml.).—Pure crystalline phenol, 1 g., is dissolved in and made to 1 litre with 0.1 N-HCl.

Working Phenol Standards.—Standard, 0.01 mg./ml. : 1 ml., stock standard, diluted to 100 ml. ; 0.03 mg./ml.: 3 ml. stock standard diluted to 100 ml. Both working standards should be preserved with 2 or 3 drops of chloroform.

N/2 Sodium Hydroxide.—Standardized.

M/2 Sodium Bicarbonate.—$NaHCO_3$, 4.2 g., dissolved in water and made to 100 ml.

4-Amino-antipyrine (A.A.P. 0.6%).—A.A.P., 0.6 g. is dissolved in water and made to 100 ml.

Potassium Ferricyanide.—$K_3Fe(CN)_6$, 2.4 g., is dissolved in water and made to 100 ml.

Methods for Alkaline and Acid Phosphatase.—These are as follows :—

Test.—Buffer, 1 ml. (*p*H 10 for alkaline, *p*H 4.9 for acid phosphatase), is added to 1 ml. substrate (M/100 Na_2-phenyl phosphate), warmed at 37° C. for three min., 0.1 ml. plasma added and mixed. The solution is incubated at 37° C., 15 min. for alkaline phosphatase (one hour for acid). Then 0.8 ml. N/2 NaOH is added for alkaline phosphatase (1 ml. N/2 NaOH for acid), and 1.2 ml. M/2 $NaHCO_3$ added for alkaline phosphatase (1 ml. M/2 $NaHCO_3$ for acid). Then 1 ml. 0.6% A.A.P. is added and mixed. Finally 1 ml. 2.4% $K_3Fe(CN)_6$ is added and mixed.

Control.—Same as test, except that plasma is added after the NaOH.

Standards.—Alkaline (or acid) buffer, 1.1 ml., + 1 ml. phenol working standard (0.01 or 0.03 mg.) + 0.8 ml. N/2 NaOH + 1.2 ml. M/2 $NaHCO_3$ for alkaline (or 1 ml. N/2 NaOH + 1 ml. M/2 $NaHCO_3$ for acid) phosphatase standards + 1 ml. 0.6% A.A.P. + 1 ml. 2.4% $K_3Fe(CN)_6$.

Blank.—Same as standard, but water is substituted for phenol.

The colours develop immediately and are stable for at least an hour if kept in dim light.

Calculation.—

$$\text{Phosphatase (K.-A. units/100 ml.)} = \frac{\text{Test}^* - \text{Control}^*}{\text{Standard}^* - \text{Blank}^*} \times 0.01 \text{ (or } 0.03) \times \frac{100}{0.1}$$

$$= \frac{\text{Test} - \text{Control}}{\text{Standard} - \text{Blank}} \times 10 \text{ (or } 30)$$

(*Photoelectric readings)

Method for Formol-stable Acid Phosphatase.—These estimations are carried out in the same manner as those for total acid phosphatase, except that 0.02 ml. of neutral 40% formalin (NaOH added until pink to phenolphthalein) is added to the buffer-substrate

TABLE V

COMPARISON OF ALKALINE AND ACID PHOSPHATASE ACTIVITIES OF VARIOUS PATHOLOGICAL PLASMAS BY THE MODIFIED A.A.P. AND KING-ARMSTRONG METHODS

	Alkaline Phosphatases		Acid Phosphatases	
	A.A.P. Method (K.-A. units)	King-Armstrong Method (K.-A. units)	A.A.P. Method (K.-A. units)	King-Armstrong Method (K.-A. units)
1	6·3	8·3	0·8	0·8
2	15·2	16·7	2·1	3·0
3	46·0	45·0	0·5	1·2
4	8·4	10·4	1·1	1·0
5	96·0	82·0	4·5	6·2
6	5·8	8·8	10·0	10·6
7	9·5	10·8	13·3	14·8
8	13·1	12·9	18·5	20·0
9	5·4	7·1	24·3	26·7
10	13·3	15·9	40·5	33·3
11	60·0	57·0	58·5	49·0
12	6·8	8·4	51·0	59·0
13	5·4	7·7	73·0	62·0
14	18·4	21·0	54·0	44·0
15	6·0	9·6	4·9	5·5
16	7·9	9·2	6·6	7·8
17	9·2	11·2	9·4	11·4
18	13·5	16·8	11·1	13·1
19	79·0	74·0	16·0	18·6
20	23·5	27·0	21·5	27·0
Average	22·43	22·99	20·11	20·75
Mean difference	2·87		3·49	
$\sqrt{\text{Mean}^2\text{difference}}$	3·99		5·03	

mixture of the test and control. The same amount of formalin is also added to the standard and blank, though this is unnecessary if a standard is already in use for total acid phosphatase, as formalin does not appear to alter the colour intensity.

Comparison of Alkaline and Acid Phosphatase Activities of Various Pathological Plasmas by Modified A.A.P. and King-Armstrong Methods

A series of alkaline and a series of acid phosphatase determinations were carried out by the modified A.A.P. method and the results compared with simultaneous determinations by the routine King-Armstrong procedure used in this laboratory (King, 1951). Table V shows that the agreement is as good as that usually obtained by enzymic methods. A comparison of results for formol-stable acid phosphatase is given in Table VI.

TABLE VI

COMPARISON OF RESULTS OBTAINED FOR FORMOL-STABLE ACID PHOSPHATASE OF PLASMAS ESTIMATED BY A.A.P. AND KING-ARMSTRONG METHODS

A.A.P. (K.-A. units)	King-Armstrong (K -A. units)
48·0	49·0
10·2	10·6
24·8	23·3
18·9	18·1
5·4	4·9
5·9	4·3
16·8	17·2
28·2	29·5
9·8	9·8
33·8	33·0
12·0	11·8
41·5	39·0
Average .. 21·2	20·9

The new method is simpler and somewhat quicker that the King-Armstrong, where precipitation and removal of proteins is necessary, and the results can be expressed in the same units.

Summary

A modified phenyl phosphate method for the determination of plasma alkaline and acid phosphatase is described. The liberated phenol is determined by reaction with 4-amino-antipyrine to yield a red-coloured quinone. This method, originally used by Grifols-Lucas, has been modified to yield results closely similar to those obtained by the King-Armstrong procedure. Results are expressed in the same K.-A. units.

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THE ENDOGENOUS CREATININE CLEARANCE IN NORMAL SUBJECTS

BY

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In normal subjects the glomerular filtration rate is relatively constant under standard conditions, and there is little or no change when the urine flow varies. It follows from this that the excretion rate of substances, which are filtered at the glomerulus and neither reabsorbed from nor secreted into the tubules, is constant. It is these substances which can be used to measure the filtration rate.

There is considerable evidence that endogenous creatinine behaves in this way, and this is supported by the data presented in this paper, which show that its excretion is very constant in spite of considerable variations in urine flow, only decreasing slightly at low urine volumes.

Methods

Four normal subjects were investigated, one on two occasions, at intervals of a year. One- to three-hour samples of urine were obtained from each subject on 28 to 48 days. Those from subjects 1 to 3 were morning specimens. Those from subject 4 were afternoon ones in experiment iv, and in experiment vi morning and afternoon ones, but the results considered separately were the same so are given combined. The minute volume of the samples was varied by dehydration or drinking.

Samples of blood were taken occasionally during each experiment.

Creatinine was estimated by the modified Jaffé reaction in urine and serum (Roscoe, 1953).

Instead of calculating the creatinine excretion rate for each sample of urine, the urinary concentrations (U) are compared with the reciprocals of the urine volume ($\frac{1}{u}$). Then if the excretion rate is constant at all urine volumes or $u.U = M$, $U = M\frac{1}{u}$ and the regression of U on $\frac{1}{u}$ is a straight line passing through zero on both axes. The rate of excretion is given by the slope of the line, M, which can be found by the method of least squares.

Alternatively, if the excretion rate is influenced by the urine flow

$$u.U = M + Nu.$$
$$U = M\frac{1}{u} + N$$

and the regression of U on $\frac{1}{u}$ is a straight line cutting the co-ordinate axis at N.

The significance of N depends on the deviation of the values about the regression line (σ y.x).

Mean creatinine clearances (f) are calculated from this expression by dividing by the filtered concentration (F), which is taken as the serum concentration.

$$f = \frac{u.U}{F} = \frac{M}{F} \quad \text{or} \quad = \frac{M + Nu.}{F}$$

The scatter of the individual values of f about the mean is then given by

$$\frac{\sigma \ M}{F}$$

Results

Creatinine Excretion Rates.—The results from subject 3, experiment iv, are shown graphically for high urine flows in Fig. 1 and for low flows in Fig. 2, where U is plotted against $\frac{1}{u}$. At low values of $\frac{1}{u}$ (high urine volumes) U is directly proportional to $\frac{1}{u}$ and the regression line of U on $\frac{1}{u}$ goes through zero or $U = M\frac{1}{u}$. In this case $M = 1{,}000$. At higher values of $\frac{1}{u}$ (low urine volumes) the relationship alters and the regression takes the form $U = M\frac{1}{u} + N$. Here $M = 706$ and $N = 293$.

The values of M and N found in each experiment are shown in Table I. In each case at high urine volumes N is not significant and the excretion rate is independent of urine flow, but at low volumes N is significant and the rate falls as the flow decreases.

Since the difference between the relationships at high and low flows is not large it is not obvious at what flow the excretion starts to be influenced by urine volume. In the same experiments the excretion of Na, Cl, K, and urea has also been studied, and the results, which it is hoped to publish later, show that a marked change in the excretion of each solute occurs when the creatinine concentration in the urine reaches a certain level. These creatinine levels, which vary in different experiments, have been taken as the dividing values and are shown in Table I. The division

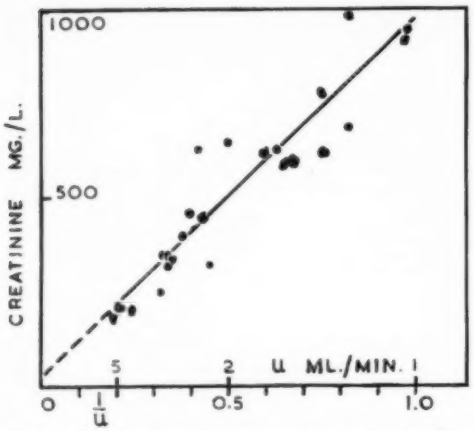

FIG. 1.—The urinary creatinine concentrations at high urine volume in subject 4, experiment iv.

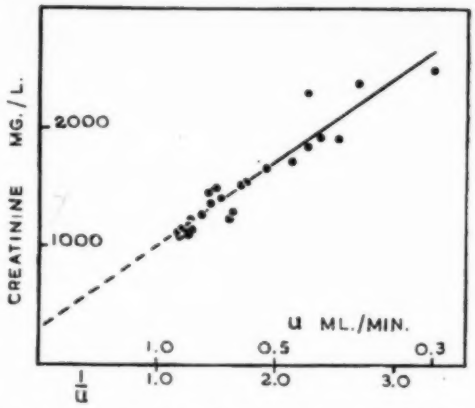

FIG. 2.—The urinary creatinine concentrations at low urine volume in subject 4, experiment iv.

could be made at a value of u, but since this is a less accurate measurement than U, especially when the urine volume is small, this is not advisable. Approximate values of u at which the change occurs are, however, given in Table I. These are means of the values calculated from the dividing values of U, and the regressions at high and low urine flows.

Creatinine Clearance.—Creatinine clearances depend on the plasma creatinine, and when in subject 3 this was estimated several times the mean values of samples obtained during dehydration were higher than those during hydration. This suggests that dehydration influences the creatinine level, either by haemoconcentration or perhaps more probably by reducing the excretion rate. There was no indication that the serum level rose progressively as the urine volume fell, so it is only possible to use mean values when these were found, and to consider that when a common value is used the clearances at high flows are perhaps too low and those at low flows too high.

The clearances in each experiment, constant at high urine flows, but falling off as the flow becomes very small, are shown in Table I. The clearances in all experiments, except subject 4, experiment iv, are higher at the upper limit of the low flow range than at high flows, but this anomaly is probably due to the filtered creatinine value used at low flows being too small, while in subject 4 this was perhaps too large.

In Table II the creatinine clearances at high flows are shown corrected to a surface area of 1.73 sq.m. and compared with the mean inulin clearances given by Smith (1951). The creatinine clearances are 20–27% lower than the expected inulin clearances.

TABLE I

THE RATE OF CREATININE EXCRETION (u.U) AT DIFFERENT URINE VOLUMES (u), THE FILTERED CREATININE CONCENTRATION (F) AND CREATININE CLEARANCE (f)

Subject	No. of Samples (n)	u (ml./min.) Range	Dividing Values u (ml./min.)	Dividing Values U (mg./l.)	u.U. = (M + uN) (µg./min.) M	σM	N	σ y.x	F (mg./l.) n	F Range	F Mean	f (ml./min.)	σ f
1	30	0·70–11·45	0·75	2,000	1,428 (1,450)	70	15*	169	3	11·6–12·6	12·0	121	6
	16	0·38– 0·74			1,150	117	519	162				43u + 96	10
2	16	0·43– 4·62	0·50	2,500	1,158 (1,185)	68	28*	173	1		10·3	115	7
	12	0·28– 0·62			1,224	78	180	114				17u + 119	8
3	23	0·71–13·03	0·81	1,500	1,080 (1,142)	55	48*	107	3	10·5–12·6	11·6	98	5
	21	0·41– 0·82			901	104	475	156				41u + 78	9
4 iv	24	1·03– 5·24	0·99	1,000	952 (1,000)	83	25*	93	3	10·8–11·7	11·2	89	7
	24	0·30– 0·85			706	45	293	124	3	12·8–14·0	13·5	22u + 52	3
vi	40	0·85– 6·70	1·07	1,000	862 (903)	46	26*	90	4	11·1–11·8	11·5	79	4
	40	0·33– 1·02			726	74	374	235	4	11·3–12·6	12·0	20u + 66	6

* These values of N are not significant. The values of M in parenthesis are those if n is taken as 0. The overlap in ranges of u at high and low flows is due to the division being made at a value of U, not u.

TABLE II
CREATININE CLEARANCES AND EXPECTED INULIN CLEARANCES

Subject and Sex	Age (Years)	Surface Area (sq.m.)	Clearances		
			Creatinine		Inulin* (ml./min. per 1·73 sq.m.)
			Actual (ml./min.)	ml./min. per 1·73 sq.m.	
1. Male..	32	2·17	121	97	127
2. „ ..	39	1·96	115	102	
3. „ ..	29	1·81	98	93	
4. Female	50	1·92	89	80	118

* Smith, 1951.

Discussion

These results show that in normal subjects the excretion of creatinine remains very constant at urine flows above about 0.5 to 1.0 ml./min., the coefficient of variation being 5–9% in different subjects, but that at lower flows the excretion rate falls. Similarly the creatinine clearance is constant at high flows, but falls at lower ones. This is in agreement with the findings of Chesley (1938), but in his subjects the fall did not take place till the urine volume fell below about 0.35 ml./min.

Qualitatively there is thus a good agreement between the clearance of creatinine and inulin, which is the most generally accepted measure of filtration rate. Many workers have found constant inulin clearances at higher flows, and Chasis and Smith (1938) found a slight decrease at low flows.

Quantitatively the agreement is not so good, the creatinine clearance being lower than the expected filtration rate measured by inulin clearance. This could be due to tubular reabsorption of creatinine, but only if a constant amount is reabsorbed whatever the urine flow. Although there is evidence that exogenous creatinine is secreted by the tubules, and it has been suggested that this may also occur with the endogenous form (Brod and Sirota, 1948), reabsorption has not been suggested, and the most probable cause of the discrepancy is that the creatinine clearances are lower than the inulin clearances because the values obtained for serum creatinine are too high. This was discussed in a previous paper (Roscoe, 1953), where it was shown that if, as has been found, 20% of the estimated chromogen is not creatinine, the difference in the clearances would be accounted for.

If this is so, although the creatinine clearances do not accurately measure the filtration rate, they do provide accurate measurements of any changes in rate which may occur, and so are valuable tools in the investigation of kidney function, especially in long-term experiments where inulin clearances cannot be used.

Summary

In four normal subjects the excretion rates of endogenous creatinine were unaffected by changes in urine volume, except when this fell to low volumes. This suggests that creatinine is not handled by the kidney tubules and that changes in its rate of excretion can be used as a measure of changes in filtration rate.

The creatinine clearance was in each case lower than the expected filtration rate, but this is considered to be due to over-estimation of the plasma creatinine.

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THE ESTIMATION OF HEPARIN AND SIMILAR SUBSTANCES IN HUMAN BLOOD AND TISSUES USING A COMBINED BIOLOGICAL AND COLORIMETRIC METHOD WITH PAPER ELECTROPHORETIC STUDIES

BY

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Heparin and other acid mucopolysaccharides were extracted from various tissues by a modification of the method previously described (Bassiouni, 1953). The dye mucopolysaccharides complex obtained was resolved into its components with formamide: the acid mucopolysaccharides were precipitated with methyl alcohol in the presence of electrolyte (sodium chloride). A solution of the purified polysaccharide precipitate was bio-assayed for active heparin, and the remainder was either submitted to electrophoresis on paper or reprecipitated with the dye and measured colorimetrically.

Methods

Reagents.—The following are required:—

(1) Sodium citrate, 3.8 g./100 ml.
(2) Sodium chloride, 0.9 g./100 ml.
(3) Sodium hydroxide, 0.2 N (Analar).
(4) Ammonium sulphate (specific grade) dried, 6.5 g.
(5) Dye solution, asymmetric dimethyl thionin or Azure A (Revector, Hopkin & Williams, Ltd.), 100 mg. dissolved in 100 ml. distilled water.
(6) Hydrochloric acid, 0.01N.
(7) Standard heparin, calcium salt of heparin, 99 units/mg. (Evans).
(8) Buffered solvent, made up of 4 parts acetone, 2 parts distilled water, 2 parts of glycine-sodium hydroxide buffer consisting of 20 parts of molar glycine solution and 80 parts of normal sodium hydroxide solution.
(9) Formamide, "pure" (L. Light & Co., Ltd., Colnbrook, Bucks).
(10) Sodium chloride, saturated solution.
(11) Methyl alcohol.
(12) Calcium chloride solution, 0.2. Molar.
(13) Phosphate buffer *p*H 7 made up of Na_2HPO_4, $2H_2O$ M/15 305.5 ml. and 194.5 ml. KH_2PO_4 M/15.
(14) Chlorobenzene.

Extraction.—Blood, 4 ml., is drawn, mixed with 1 ml. 3.8% sodium citrate, and centrifuged at 2,000 r.p.m. for 10 minutes: the plasma is then pipetted off, the cells washed with 2 ml. saline, and the wash added to the plasma. The plasma is made up to 7 ml. with distilled water in a conical pyrex flask of 25 ml. capacity; the white cell layer is pipetted off and suspended in another conical flask of the same capacity and containing 7 ml. distilled water. For tissue analysis, 1 g. of fresh tissue is weighed out, then cut into small pieces, macerated with powdered glass and transferred with 7 ml. distilled water into a pyrex conical flask of 25 ml. capacity.

To each 25-ml. flask, 0.5 ml. sodium hydroxide 2N is added; the flask is corked and warmed at 50° C. in a hot-water bath for 20 to 30 minutes with shaking from time to time; 6.5 g. ammonium sulphate is then added, the flask recorked, shaken vigorously, and left for five minutes. It is then warmed quickly in a boiling-water bath to 75° C., vigorously shaken and the contents transferred rapidly to fluted filter paper (Whatman 1, 7 cm. diameter). The filtrate is received into a thoroughly cleaned conical centrifuge tube of 12 ml. capacity, containing 1 ml. distilled water and 1 ml. dye solution. The tube is corked and inverted three times to mix, then left for an hour, being shaken from time to time; finally it is centrifuged for 45 minutes at 3,000 r.p.m. while capped. It is then decanted, the residue being washed first with 5 ml. 0.01N HCl and then with 2 ml. distilled water. Now the precipitate is resolved into its components by dissolving in 0.25 ml. formamide, helped by warming to 50° C. for a few minutes; it is then centrifuged and the supernatant liquid taken off with a micropipette and transferred to a clean tube. To it is added 0.25 ml. of saturated solution of sodium chloride and 2 ml. methyl alcohol, the mixture being centrifuged at 3,000 r.p.m. for 20 minutes.

The precipitate is re-extracted with 0.25 ml. saturated solution of sodium chloride; to the extract 0.25 ml. distilled water and 2 ml. methyl alcohol

are added and the mixture centrifuged for 20 minutes. The tube is decanted for five minutes, the mouth wiped dry and the tube dried *in vacuo*. The precipitate is dissolved in 0.5 ml. distilled water and 0.1 ml. of the solution used for bio-assay to which 1 ml. plasma is added.

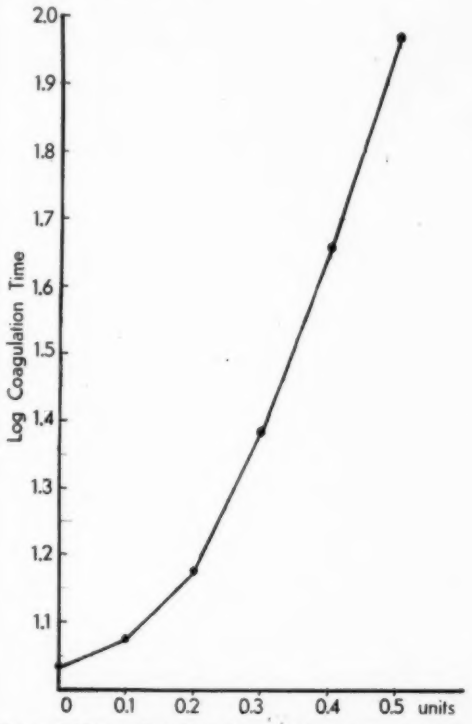

FIG. 1.—Inhibitory effect of heparin on coagulation of human plasma.

Biological Estimation.—The biological estimation is based on an observation that within a certain range of concentration of heparin added to re-calcified sheep plasma, the logarithm of the clotting time is proportional to the amount of heparin added (Fig. 1). Fresh sheep blood collected from the slaughter-house, 400 ml., is received in a glass flask containing 100 ml. 3.8% sodium citrate and the plasma is separated and kept in 20-ml.-capacity bottles in a refrigerator. The amount of calcium chloride M/5 solution necessary for optimum recalcification of 1 ml. plasma is determined.

The heparin clotting inhibition curve is obtained by preparing a series of conical tubes (thoroughly cleaned, 12 ml. capacity) each containing 1 ml. of sheep plasma.

To one tube is added 0.1 ml. distilled water to serve as a blank; to the rest is added 0.1 ml. solution of standard heparin in distilled water containing amounts varying from 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 international units. The tubes are corked and kept in a water bath at 25° C. for 10 minutes, then 0.2 ml. of M/5 calcium chloride solution is added to each tube and clotting observed every half minute. The method depends on recording the clotting time at a certain fixed stage in all the tubes. This is taken to be such that when the tube is tilted to an angle of 45° the surface of the plasma does not tilt at all, keeping strictly at right angles to the tube. The logarithm of clotting time is obtained and plotted against the amount of heparin added.

Human plasma from stored blood whose date of use has expired (30 days) can be used for the same purpose. It is noteworthy that the older the samples, whether sheep or human, the more sensitive they are to the action of heparin, most probably due to the fact that prothrombin concentration declines with storage, especially with rise of temperature, leading to less thrombin on recalcification. Thus the antithrombin action of heparin becomes more apparent. Fresh human plasma was found insensitive to such low concentrations of heparin.

Estimation of Unknown Sample.—The heparin content of the unknown sample is read off on the heparin inhibition curve. When the unknown gives a higher clotting time than the standard tubes, 0.05 ml. of unknown solution is made up to 0.1 ml. with distilled water and assayed again. Confirmatory experiments to assess the efficacy of this method of assay were done by comparing part of an extract, whose biological activity had been determined, with a known standard of the same strength. Both gave exactly the same clotting time. For each experiment a standard curve should be done.

Colorimetric Estimation.—A standard curve is prepared by mixing 1 ml. of a diluted standard solution of heparin containing from 0.01 mg. up to 0.1 mg. with 1 ml. dye solution. This is then centrifuged for 30 minutes and the precipitate is washed successively with 5 ml. 0.01 N HCl and 2 ml. distilled water, then dissolved in 8 ml. of buffered solvent and is estimated electro-colorimetrically in a single cell spectrophotometer using a blue-green filter No. 623.

Estimation of Unknown Sample.—Of a dilution of the extract, 1 ml. is mixed with 1 ml. dye solution and the same procedure followed as for the standard curve.

Paper Electrophoresis of the Extract.—Electrophoresis of heparin and chondroitin sulphate on filter paper was carried out, using the apparatus and following the general procedure described by Consden and Stanier (1952). Whatman No. 1 filter paper was used with phosphate buffer *p*H 7 as the electrolyte and chlorobenzene as the organic solvent. The unknown heparin extract, the standards of heparin, and the chondroitin sulphate were each dissolved in 0.02 ml. distilled water, and applied to the filter paper as a thin band on the same level 0.5 cm. from the end near the cathode. The voltage was 300 v and the potential gradient approximately 7.3 v/cm. applied

for two hours, after which the paper was removed and dried at 100° C. It was then sprayed with an alcoholic solution of Azure A 0.033% w/v in methyl alcohol. (Chondroitin sulphate was kindly supplied by Dr. E. J. Holborrow prepared from human cartilage by the method of Einbinder and Schubert, 1950.)

Staining with an alcoholic solution of the dye gave bands with clear, well-defined margins, whereas staining with an aqueous dye solution gave bands with ragged and ill-defined margins (Consden, 1953).

Recoveries

The recoveries and losses at various stages of the procedure were studied in detail by extracting a known amount of heparin from a blank reagent. The results (Tables I, II, Fig. 2) show that heating,

TABLE I

RECOVERIES OF 70 μG. HEPARIN (7 UNITS) AT TWO STAGES OF PROCEDURE

Procedure	Recovery at Stage of Obtaining Heparin-dye Complex (μg.)	Recovery (Biological Method) after Removal of Dye and Precipitation of Heparin (units)
Extraction from plasma	50–47	2·5
In blank undergoing whole procedure ..	52	3·1
In blank with no NaOH added	50	3·0
In blank with no heating and no filtration ..	60	3·3

TABLE II

PERCENTAGE OF ADDED HEPARIN RECOVERED AT THE TWO STAGES OF PROCEDURE

Added Heparin		Recovery at Stage of Obtaining Heparin-dye Complex		Recovery after Removal of Dye and Precipitation of Heparin with Methyl Alcohol	
μg.	Units	μg.	%	Units	%
1·0	0·1	0·7	70	0	0
2·0	0·2	2·0	100	0	0
3·0	0·3	3·0	100	0	0
4·0	0·4	4·0	100	0–0·05	0–12
5·0	0·5	5·0	100	0·17	32
6·0	0·6	5·4	90	0·21	35
10·0	1·0	9·0	90	0·34	34
30·0	3·0	22·0	70	1·0	33
50·0	5·0	35·0	70	1·6	33
60·0	6·0	45·0	80	2·2	37
70·0	7·0	50·0	80	2·5	36
100·0	10·0	80·0	80	5·0	50

filtration, and washing the heparin-dye complex cause a total loss of about 15% whether 0.5 ml. of 2N NaOH were added or not ; with extraction of the same amount of heparin from the 4 ml. of stored plasma the loss rises to 20%, i.e., 5% more than in the blank reagent. About half of the remaining 80% (40% of the total) is lost during the

TABLE III

VARIOUS VALUES FOR STANDARD HEPARIN AND CHONDROITIN SULPHATE

	Anti-coagulant Units	Colorimetric Value (mg.)	A/C Value*	Paper Electrophoresis	
				Mobility	Colour of Stained Bands
1 mg. heparin ..	100	1·0	100	100	Alpha form
1 mg. chondroitin sulphate ..	0–2	0·5	0–4	87·2	Beta form

* A/C value = $\frac{\text{anticoagulant activity in units}}{\text{colorimetric value in mg.}}$

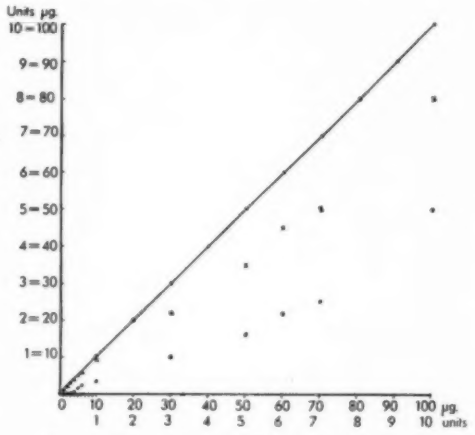

FIG. 2.—Recoveries: colorimetric × and biological ●.

formamide separation of heparin from the dye. This leaves us with 40 to 50% of the starting material ; the proportion of this loss is so constant within the range 1–20 units that it may be allowed for in the calculation of the result without serious error. The figures given in Tables V and VI are corrected ones, the directly obtained values being multiplied by 2 for both the anticoagulant and the colorimetric estimation in order to allow for the inevitable loss. This loss in the last step is a material one as reprecipitation with the dye proves. It seems inevitable with very low concentration of heparin, and efforts to achieve higher recovery were not successful. The method cannot detect an initial concentration of heparin less than 0.5 units/2 ml. (5 μg./2 ml. plasma) since, by the stage of freeing the heparin from the dye, the 0.5 unit becomes 0.17 unit (1.7 μg.) which is the minimum amount detectable biologically.

TABLE IV

VARIOUS VALUES FOR ADULT AND NEWLY BORN HUMAN TISSUES

Tissue	Anti-coagulant (Units/1 g. Tissue)	Colori-metric Value (mg./1g. Tissue)	A/C Value	Paper Electrophoresis	
				Mobility	Colour of Wet Stained Band
*Adult human:					
lung	11·0	0·118	92·0		
heart	4·2	0·070	60·0		
liver	3·9	0·070	55·0		
spleen	3·3	0·057	58·0		Gamma-beta forms
kidney	10·25	0·118	87·0	94·7	
brain	2·4	0·040	60·0		
†Connective tissue..	5·3	0·360	15·0		
Cartilage	2·75	0·600	4·6		
Aorta	1·8	0·280	6·3		
‡Severely atheroma-tous aorta ..	1·6	0·190	8·3		
‖Blood, 450 ml.:					
R.B.C.s	0	0	0	84–87·2	Beta forms
W.B.C.s	0·8	0·080	10·0		
Plasma	0	0	0		

* From a woman aged 60: sudden death (ruptured heart).
† From fibrous capsule of knee-joint.
‡ From a case of diabetes and acromegaly.
‖ From a one-week-old blood from the transfusion centre.

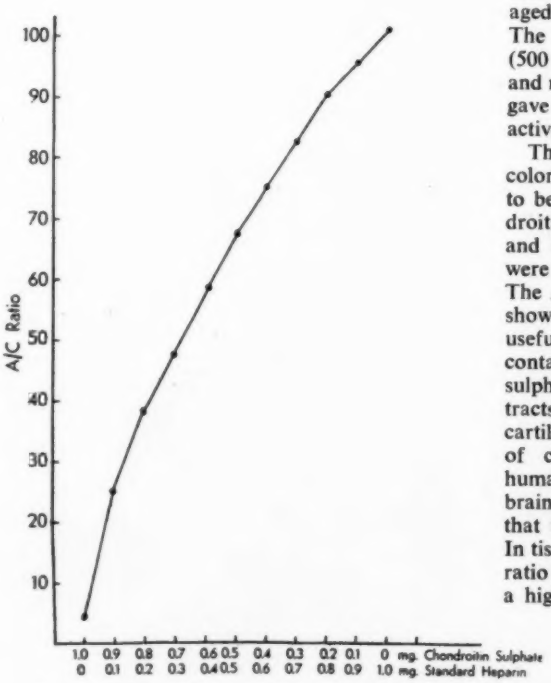

FIG. 3.—The values of the A/C ratio for various mixtures of heparin and chondroitin sulphate.

Results

The estimation of heparin-like substances in various adult and newly born tissues are shown in Tables IV and V. The highest content in the adult was found in the kidneys and lungs, being about three times the content in other tissues. In the newly born the concentration is low and shows nearly similar values in all the organs analysed.

TABLE V

RESULTS OF ESTIMATIONS IN TISSUES OF THE NEWBORN*

Tissues	Anti-coagulant (Units)	Colori-metric Value (mg.)	A/C Value	Paper Electrophoresis	
				Mobility	Colour of Wet Stained Band
Lung ..	2·5	0·100	25·0		
Heart ..	2·5	0·120	20·8		
Liver ..	2·1	0·100	21·0	90	Beta form
Spleen ..	2·6	0·080	32·5		
Kidney	2·5	0·080	31·2		

* From a case of complete spina bifida.

The plasma from 4 ml. blood samples taken from healthy adults (10 men and four women, aged 20–66) gave negative results biologically. The extraction of the larger amounts of blood (500 ml.) also gave negative results for both plasma and red blood cells; the white blood cells, however, gave an extract with very low anticoagulant activity and high colorimetric value.

The ratio of anticoagulant activity in units to the colorimetric value in mg. (A/C ratio) was found to be 100 for standard heparin and 0–4 for chondroitin sulphate (Table III). Mixtures of heparin and chondroitin sulphate in various proportions were estimated biologically and colorimetrically. The A/C ratio obtained for various mixtures was shown to be additive (Fig. 3). This ratio is very useful in indicating whether the obtained extracts contain mainly heparin-like or chondroitin sulphate-like substances. The A/C ratio in extracts from the aorta, white blood cells, and cartilage ranges between 6 and 10, similar to that of chondroitin sulphate, while extracts from human adult kidney, lung, heart, liver, spleen, and brain gave a ratio between 60 and 90, suggesting that they contain mainly heparin-like substances. In tissues from human newly born infants the A/C ratio was from 25 to 30, which would either mean a higher percentage of chondroitin sulphate or a heparin with a low anticoagulant activity.

On paper electrophoresis the band of chondroitin sulphate had a mobility of 87.2 relative to that of standard heparin as 100. The "heparin" extracted from

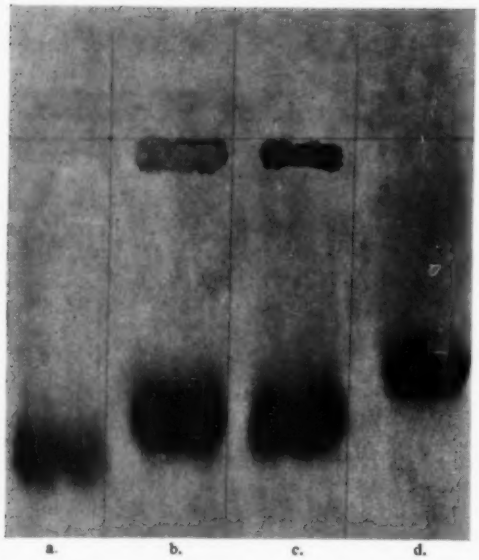

FIG. 4.—Electrophoresis of heparin from adult human kidney and spleen.
a.=standard heparin, 30 μg. b.=heparin from kidney.
c.=heparin from spleen. d.=chondroitin sulphate, 120 μg.

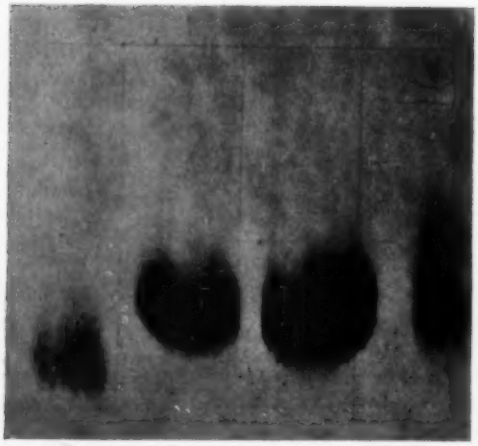

FIG. 5.—Electrophoresis of heparin from a newly born human kidney and heart.
a.=standard heparin. b.=heparin from heart.
c.=heparin from kidney. d.=chondroitin sulphate, 120 μg.

human adult kidney, lung, heart, liver, spleen, and brain occupied an intermediate position between standard heparin and chondroitin sulphate (Fig. 4). The "heparin" from tissues of the newly born infant occupied a position much nearer to the chondroitin sulphate band (Fig. 5) while the adult aorta and white blood cell extracts gave bands on the same level as chondroitin sulphate (Fig. 6).

The metachromasia of the stained bands on filter paper while wet showed also distinct differences; the band of standard heparin was red, that of human adult heparin was reddish violet, while that of the newly born heparin was violet, as well as that of chondroitin sulphate.

The metachromasia was ascribed by Lison (1936) to the sulphate content of the mucopolysaccharide, and later thought by Michaelis and

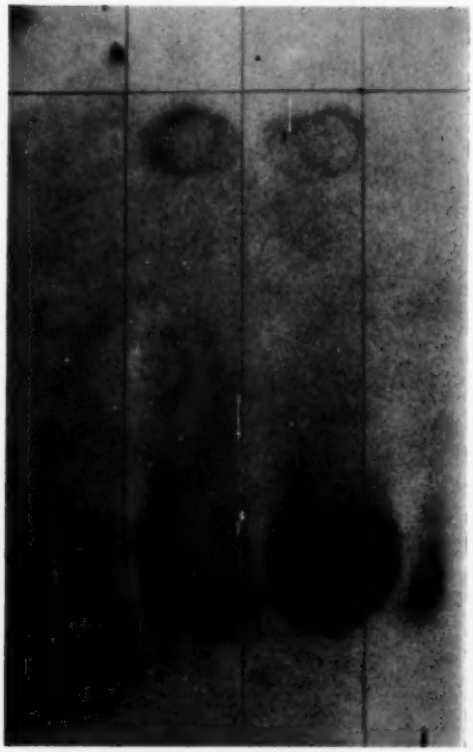

FIG. 6.—Electrophoresis of aortic extracts on filter-paper.
a.=standard heparin, 25 μg. b.=extract from normal aorta.
c.=extract from an atheromatous aorta. d.=chondroitin sulphuric acid, 25 μg.

Granick (1945) and Michaelis (1947) to be due to the degree of polymerization of the dye when combined with various acid mucopolysaccharides, being red in trimers γ-form, violet in dimers β-form, and blue in monomers α-form.

These differences therefore suggest the possibility that the heparin-like substances of the tissues in the newly born are less esterified or less polymerized than in the adult. A higher percentage of chondroitin sulphate, as suggested by the A/C ratio, cannot explain the mobility differences. The electrophoretic results suggest that extracts of cartilage, aorta, and white blood cells contain a substance, or substances, with properties similar to chondroitin sulphate.

Summary

A micro-method is described for the extraction of heparin and chondroitin sulphate from 1 g. of various human tissues and their estimation biologically and colorimetrically. The ratio of anticoagulant activity in units to the colorimetric value in mg. (A/C ratio) can indicate whether the extract is heparin or chondroitin sulphate or a mixture of both.

Extracts were also run on paper electrophoresis giving additional information from the rate of mobility and the colour of stained bands.

The results of estimations of extracts from adult and newly born tissues showed distinct differences between them in their heparin content, A/C ratio, and electrophoretic mobility.

My thanks are due to the Egyptian Government for a grant which enabled me to conduct this work.

I wish to express my thanks to Dr. E. G. L. Bywaters and Dr. L. E. Glynn for their interest and helpful criticism of the work.

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THE DYE-BINDING CAPACITY OF HUMAN PLASMA DETERMINED FLUORIMETRICALLY AND ITS RELATION TO THE DETERMINATION OF PLASMA ALBUMIN

BY

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The binding of acidic dyes and other negatively charged ions is a well-known property of the proteins of blood plasma and is a specific property of the albumin fractions, the globulin fractions being almost inactive in this respect. This is easily observed when an acidic dye such as bromophenol blue is added to plasma and the plasma then subjected to electrophoresis on filter paper. The blue dye travels solely with the plasma albumin, leaving the globulin fractions unstained. A similar result is observed with the naturally occurring bile pigments in jaundiced plasma. Much further evidence is available for this specific property of plasma albumin and has been reviewed by Goldstein (1949), by Klotz (1949), and by Edsall (1947).

Smith and Smith (1938) showed that for a number of plasmas from pathological as well as normal states the retention of phenol red by an ultrafiltration membrane was closely related to the albumin content of the plasma. Rosenfeld and Surgenor (1952) have proposed a method of estimation of human plasma albumin in whole plasma by using the interaction with haematin, the extent of the interaction being measured spectrophotometrically.

In the present paper, the substance 1-anilinonaphthalene-8-sulphonic acid is used to estimate the dye-binding capacity of human plasma from a number of pathological conditions. The substance is non-fluorescent in aqueous solution, but brightly fluorescent when absorbed by plasma albumin and is one of a class of compounds with this unusual property described elsewhere (Weber and Laurence, 1954). The method to be described depends on the use of a limited amount of plasma, so that the fluorescent substance is always present in excess of the dye-binding capacity. The amount of dye bound is then a measure of the dye-binding capacity of the plasma and it is evaluated from the fluorescent intensity as only the bound dye is fluorescent. By using standard solutions of a purified albumin to relate albumin concentration with dye-binding capacity, the fluorescent intensities may be converted to equivalent albumin concentrations. Comparisons between these estimates and results of independent determinations by standard techniques are given. As no generally accepted absolute method of albumin determination in plasma is available, the best criterion of the validity is statistical agreement between any two independent methods. This criterion is used to assess the fluorimetric methods and also the standard methods of albumin determination.

Fluorimetric Methods

Reagents.—The following reagents were used in the fluorimetry:—

Fluorescent Standard Solution.—1-Naphthylamine-6(7)-sulphonic acid (B.D.H.), 40 mg., was dissolved in 100 ml. of 0.1 M-borate buffer (*p*H 9.2) and diluted to 1 litre with water.

Stock Dye Solution.—1-Anilinonaphthalene-8-sulphonic acid was prepared by the method of Hodgson and Marsden (1939) and 4 mg. was dissolved in an equivalent amount of 0.1N-NaOH and diluted to 500 ml. with water.

The 1-anilinonaphthalene-8-sulphonic acid was prepared by heating together for 15 hours at 150° C. a mixture of 1-naphthylamine-8-sulphonic acid (15 g.), aniline (23 g.), and aniline hydrochloride (9 g.) in a flask fitted with mechanical stirring and a reflux condenser. The product was extracted with sodium hydroxide and the extract was acidified with acetic acid. Impurities of a deep brown colour were removed by treating the solution with activated charcoal and the purified sulphonic acid was then crystallized by the addition of concentrated hydrochloric acid.

Human Plasma Albumin Solution.—The crystalline protein (Massachusetts Biologic Laboratories, obtained from the Smith, Kline and French Laboratories, Philadelphia, as a gift) was dissolved in isotonic saline to give a 4% (w/v) solution.

Bovine Plasma Albumin Solution.—The crystalline protein (Armour and Company, Ltd.) was dissolved in isotonic saline to give a 4% (w/v) solution.

Phosphate Buffer.—Six volumes of M/15 disodium hydrogen phosphate were mixed with 4 volumes of M/15 potassium dihydrogen phosphate.

Isotonic Saline.—Sodium chloride solution, 0.9% (w/v), in water.

Solution A.—For the estimation of dye-binding capacity, 250 ml. of the stock dye solution were mixed with 50 ml. of the phosphate buffer and 50 ml. of isotonic saline. This mixture is called "Solution A."

The Fluorimeter.—In early work the fluorescent intensity was measured visually using a modified Pulfrich photometer. The procedure to be described was adapted for use with a photoelectric fluorimeter in order to eliminate subjective errors.

The photoelectric measurement of fluorescent intensity was made using a photomultiplier type R.C.A. 931-A operated from a number of 120 v. high tension dry batteries. A Pye "scalamp" galvanometer with a linear scale was used as the measuring instrument. A G.E.C. 80 watt mercury lamp, type MB/V, with a clear glass envelope provided the light for excitation of the fluorescence which was passed through a Chance light filter OX 7 before entering the fluorimeter. The fluorescent solutions were placed for measurement in parallel-sided cells (4 cm. × 2 cm. × 1 cm. internal dimensions) illuminated on a larger face. The fluorescent intensity was measured in a direction at right angles to the direction of excitation by the photomultiplier, provided with a Chance OY 18 light filter.

Procedure.—Into 7 ml. of solution A was pipetted 0.05 ml. of the plasma to be tested and the solution was well mixed. Into another 7 ml. of solution A was pipetted 0.05 ml. of either the human or the bovine plasma albumin solutions.

The voltage across the photomultiplier was adjusted until the fluorescent intensity of the fluorescent standard solution corresponded to 67 galvanometer scale units (full scale deflection = 140 units). This adjustment was subsequently checked between each pair of fluorimetric readings.

The fluorescent intensity of the solution A with added plasma albumin solution was measured and confirmed to be either 70 (human albumin) or 90 (bovine albumin).

The fluorescent intensity of the solution A with added test plasma was measured and the corresponding albumin concentration obtained from a standard curve. This standard curve had previously been constructed by adding varying volumes of the human plasma albumin solution to 7 ml. of solution A.

The albumin concentration so obtained was compared with the albumin concentration estimated by independent methods.

Methods of Albumin Estimation

The estimates of plasma albumin concentration used in this work were mainly obtained during routine analysis of hospital samples. The methods used were three routine methods available at various times during the past two years.

These methods were as follows: (*a*) The salt fractionation procedure of Campbell and Hanna as described by King (1951) using sodium sulphite; (*b*) electrophoresis on filter paper as described by Knedel (1951) using the electrophoresis chambers and dye (amidoschwarz 10 B) supplied by Bender and Hobein (Munich) and a commercial densitometer. A washing solution consisting of 10% (v/v) glacial acetic acid in methanol (2 parts) diluted with water (1 part) and 0.01 N with respect to hydrochloric acid (Herbert, D., unpublished observations) was used to remove the background colour from the dyed filter paper strips. (*c*) Electrophoresis on filter paper in which the recording densitometer (Laurence, 1954) was used for evaluating the dyed strips. Procedure otherwise as in (*b*).

In methods (*b*) and (*c*) the total protein concentration is required and was estimated by the Van Slyke copper sulphate method as described by King (1951).

(*d*) In addition to these three routine methods, a method of paper electrophoresis was used for which the evaluation of the albumin concentration is independent of assumptions concerning the relative densities of the dyed protein fractions and does not require a knowledge of the total protein concentration.

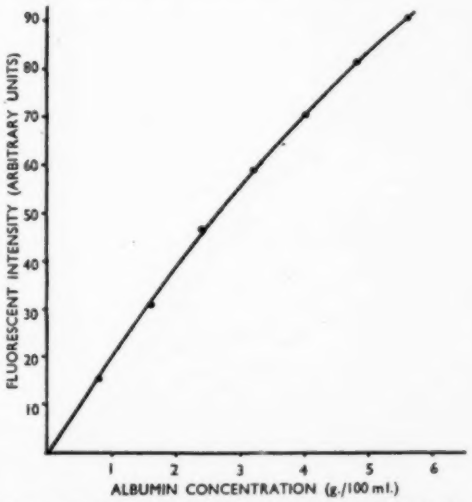

FIG. 1.—Standard curve relating the fluorescent intensity of the dye-protein mixture with the albumin concentration.

A 4% solution of the human plasma albumin was applied to the filter paper strip as a narrow band about 2 inches in advance of the test plasma, the volumes of both the albumin solution and the test plasma being exactly 10 μl. After electrophoresis the dried filter paper strip was developed with bromphenol blue as described by Kunkel and Tiselius (1951), and the dyed bands corresponding to the albumin of the standard solution and of the test plasma were cut out, eluted, and compared colorimetrically.

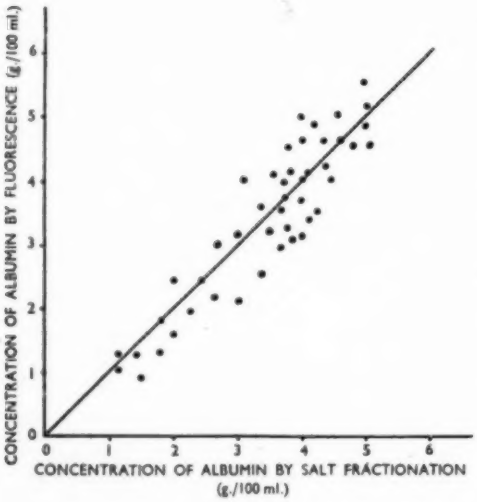

FIG. 2a

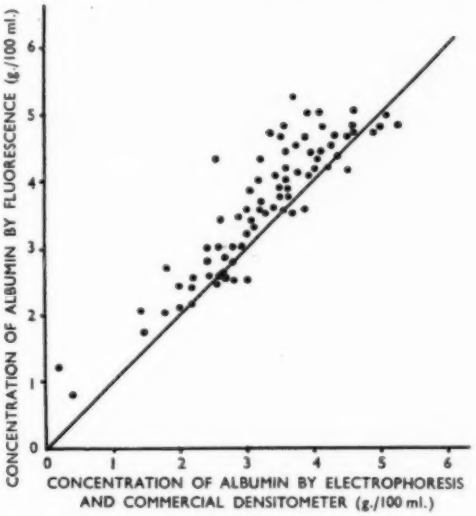
CONCENTRATION OF ALBUMIN BY FLUORESCENCE (g./100 ml.)
CONCENTRATION OF ALBUMIN BY ELECTROPHORESIS AND COMMERCIAL DENSITOMETER (g./100 ml.)

FIG. 2b

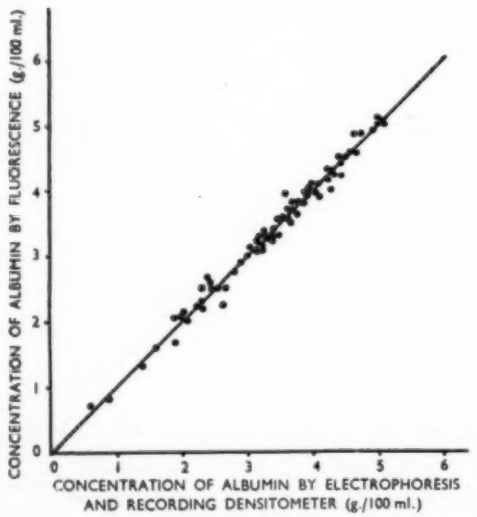
CONCENTRATION OF ALBUMIN BY FLUORESCENCE (g./100 ml.)
CONCENTRATION OF ALBUMIN BY ELECTROPHORESIS AND RECORDING DENSITOMETER (g./100 ml.)

FIG. 2c

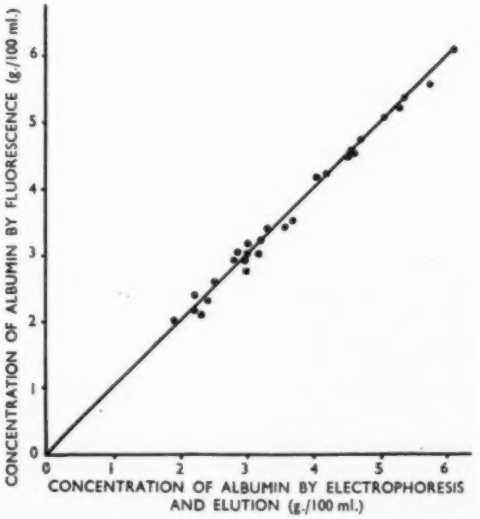

FIG. 2d

FIG. 2.—Comparisons of the albumin concentration determined fluorimetrically with the albumin concentrations determined by: (*a*) salt fractionation, (*b–d*) electrophoresis, (*b*) a commercial densitometer, (*c*) the recording densitometer, (*d*) the elution method. Curves *a–d* correspond to methods *a–d* described in the text.

Results

Fig. 1 shows the standard curve by which the apparent albumin concentration may be estimated from the values of fluorescent intensity.

Figs. 2*a–d* give the results obtained by comparing the apparent albumin concentrations obtained fluorimetrically with the values obtained by the methods (*a–d*) outlined above. The values obtained for samples of plasma from cases of obstructive and hepatic jaundice have been shown separately in Fig. 3 since these require special consideration.

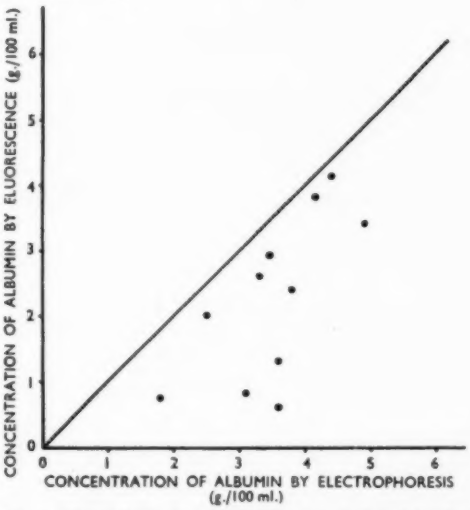

FIG. 3.—Comparisons of the albumin concentration determined fluorimetrically with the albumin concentration determined electrophoretically in cases of obstructive and hepatic jaundice.

Table I summarizes the distribution of differences irrespective of sign between the fluorimetric method and the various independent methods of estimating the albumin concentration. It will be seen that methods (*c*) and (*d*) give by far the most satisfactory agreement between the methods. The errors observed are no more than would be expected from apparent limitations of the methods and no systematic error is discernible in Figs. 2*c* and 2*d*.

Discussion

As the methods described here for electrophoresis on filter paper utilize a form of binding acidic dyes, the use of these methods as an independent check on the fluorimetric method requires justification. The dye binding in neutral aqueous solution as measured by the fluorimetric method is a specific property of the native albumin

TABLE I

DISTRIBUTION OF DIFFERENCES BETWEEN ALBUMIN CONCENTRATION DETERMINED FLUORIMETRICALLY AND BY OTHER METHODS*

Method	Differences between Estimated Concentration (g./100 ml. to nearest 0·1 g.)						Average Difference (g./100 mL.)
	0	0·1	0·2	0·3	0·4	> 0·4	
(*a*) Salt fractionation ..	11	18	10	12	5	27	0·3
(*b*) Electrophoresis and commercial densitometer	13	25	21	18	15	30	0·3
(*c*) Electrophoresis and recording densitometer	29	33	22	16	3	0	0·1
(*d*) Electrophoresis and elution	10	12	4	2	0	0	0·1

* Each entry gives the number of cases with a given difference.

fraction. It is destroyed by heating the solution at 100° for 15 minutes and is related to a maximum capacity of about 3 molecules of dye bound for each molecule of protein. In contrast, the dye binding on proteins in paper electrophoresis is a general property of all coagulated proteins in an acidic dye bath. Heating at 105° for 15 minutes is a standard procedure to promote dye binding, and more than 20 molecules of dye are bound for each molecule of protein. It is likely, therefore, that the two methods are independent. This conclusion is strengthened by the good agreement that has been obtained by many workers between the results of electrophoresis on filter paper and by the classical moving boundary method of Tiselius, the latter method depending on refractive index measurement to obtain an estimate of protein concentration.

Good agreement has been found between the plasma albumin concentration and the dye-binding capacity of the plasma, when this is expressed in terms of the dye-binding capacity of crystalline human plasma albumin (Figs. 2*c* and 2*d*). Since the dye-binding capacity is readily measured, it has formed a valuable check on the methods of protein estimation which have been used routinely.

The dye-binding capacity was consistently low in cases of obstructive and hepatic jaundice (Fig. 3), but the possibility of an artifact due to absorption of the fluorescent light by the bile pigments has not yet been ruled out. No other diseases associated with the characteristically abnormal dye-binding capacity of the plasma have been recognized, although the cases illustrated in Figs. 2*a–d* include a random and miscellaneous collection of disease including nephrosis and nephritis.

By comparison of the various Figs. 2*a–d* some typical errors in some methods of albumin determination can be recognized. The wide scatter of the results of salt fractionations (Fig. 2*a*) and the

high values for albumin, especially at low concentrations, have been recognized elsewhere (Pillemer and Hutchinson, 1945). The commercial densitometer gave results leading to an underestimation (Fig. 2*b*) of the albumin concentration, possibly owing to stray light leaking at the edges of the protein bands. In contrast, the recording densitometer (Fig. 2*c*) and the elution technique (Fig. 2*d*) both lead to satisfactory agreement with the fluorimetric determinations. In a series of normal plasmas (Kind, Rees, and Schmidt, unpublished data) a similar good agreement between the fluorimetric and the densitometric techniques was obtained.

The fluorimetric technique is especially simple to use since the fluorescent intensity of the 1-anilinonaphthalene-8-sulphonic acid is negligible in the absence of albumin and almost linear with albumin concentration. The results of this work confirm that purification and crystallization do not alter the dye-binding capacity of human serum albumin.

Summary

The dye-binding capacity of plasma has been measured in a random selection of pathological cases. There is a close, almost linear, relationship between the dye-binding capacity and the concentration of the plasma albumin.

Low values of dye-binding capacity are obtained in obstructive and hepatic jaundice.

The application of this method to check routine methods of protein estimation is illustrated.

The authors would like to thank Professor E. J. King and Dr. G. Weber for helpful encouragement.

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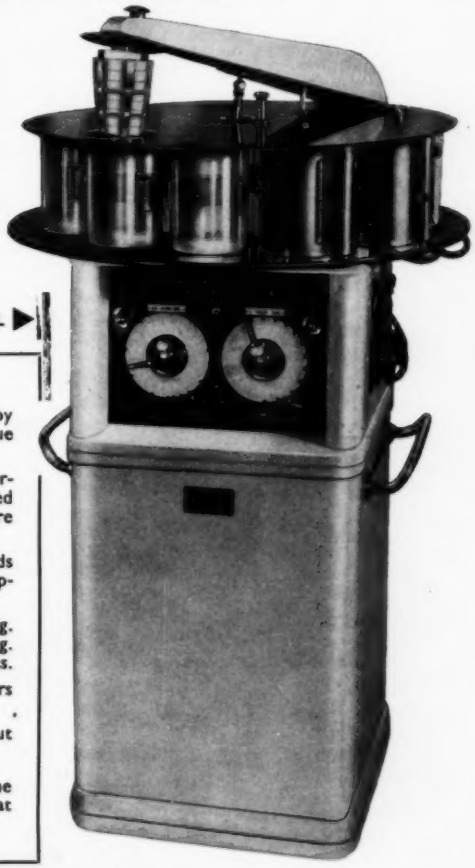

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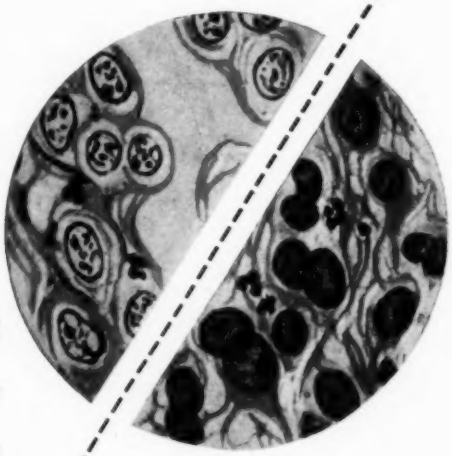

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SERUM 5-NUCLEOTIDASE

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Reis (1934, 1940, 1951) has demonstrated the presence of alkaline phosphatase in various tissues specifically hydrolysing 5-nucleotidase such as adenosine and inosine-5-phosphoric acids. The enzyme has its optimum action at *p*H 7.8 and in all human tissues except intestinal mucosa its activity, at the physiological *p*H range, is much more pronounced than that of the non-specific alkaline phosphatase. Thus ossifying cartilage, one of the classical sites of high alkaline phosphatase activity, although very active against say phenyl phosphoric acids at *p*H 9, is more active against adenosine-5-phosphoric at *p*H 7.5. This finding suggested to Reis that the enzyme might play a part in calcification mechanisms. Studies designed to elucidate the physiological significance of phosphatase are frequently based on measurements of enzyme activity in blood serum or plasma, and there is an enormous literature dealing with such variations caused by different pathological conditions. It seemed, therefore, of interest to determine the activity of both serum-5-nucleotidase and non-specific alkaline phosphatase in a number of disease states.

Reagents

Glycerophosphate Buffer Substrate pH 9.3.—Sodium β glycerophosphate, 0.05 g., sodium barbitone 0.424 g. made up to 100 ml. with water, adjusted to *p*H 9.3 and stored in the cold.

Glycerophosphate Buffer Substrate pH 7.5.—As above except that the *p*H is adjusted to 7.5 by adding approximately 1.2 ml. N HCl.

Adenosine-5-phosphate Buffer Substrate pH 7.5.—Adenosine-5-phosphate, 0.087 g., and sodium barbitone, 0.424 g., made up to 100 ml. with water and the *p*H adjusted to 7.5 by adding 1.2 ml. N HCl and stored in the cold.

Aminonaphtholsulphonic Acid Reagent.—Fifteen per cent. sodium bisulphite, 195 ml., 1,2,4-aminonaphtholsulphonic acid, 0.5 g., 20% sodium sulphite, 5 ml., stoppered and shaken until dissolved and stored in the cold and used within four weeks.

Molybdate Reagent.—Reagent grade ammonium molybdate, 25 g., dissolved in about 200 ml. water. In a 1-litre volumetric flask place 300 ml. 10 N H_2SO_4, add the molybdate solution, and dilute with washings to 1 litre with water.

Standard Phosphate Solution.—KH_2PO_4 0.02195 g., and 50 g. trichloracetic acid, made up to 1 litre. This solution contains 0.02 mg. P in 4 ml.

Methods

Non-specific alkaline phosphatase activity is high at *p*H 9 towards phenylphosphate adenosine phosphate and glycerophosphate, in this decreasing order (cf. Reis, 1951). At *p*H 7.5, however, the total phosphatase activity is equally low towards phenyl- or glycero-phosphate and the higher activity towards adenosine-5-phosphate at this reaction is reasonably inferred to be due to the specific 5-nucleotidase. Serum 5-nucleotidase activities were thus measured by subtracting the non-specific phosphatase activity with glycerophosphate as substrate at *p*H 7.5 from the total activity with adenosine-5-phosphate at 7.5. Serum non-specific phosphatase activities were determined by incubation with glycerophosphate at *p*H 9.3. Thus for the test solutions 0.2 ml. serum was mixed with 4.5 ml. of the appropriate combined buffer substrate mixture and 0.3 ml. of water and incubated at 37° C. for two and a half hours. Then the tubes were removed from the incubator and immediately mixed with 1 ml. of 30% trichloracetic acid, allowed to stand for a few minutes, and filtered. In another tube a control is prepared in an exactly similar manner without incubation. To 4 ml. each of the trichloracetic filtrates and standard phosphate solution are added 0.5 ml. of molybdate reagent, 0.2 ml. aminonaphtholsulphonic acid reagent, and 0.3 ml. of water. After 15 minutes the colours are read in the spectrophotometer at 660 mμ.

Calculation

Adenosine-5-phosphate buffer substrate *p*H 7.5 = A
Glycerophosphate buffer substrate *p*H 9.3 = G 9.3
Glycerophosphate buffer substrate *p*H 7.5 = G 7.5

$$\text{Alkaline phosphatase (units/100 ml.)} = \frac{\text{Reading of G 9.3 (test} - \text{control)}}{\text{Reading of standard}} \times 6$$

$$\text{5-nucleotidase (" units "/100 ml.)} = \frac{\text{Reading of A (test} - \text{control)} - \text{Reading of G 7.5 (test} - \text{control)}}{\text{Reading of standard}} \times 6$$

The alkaline phosphatase activities are thus in Bodansky units and the method is essentially that of Shinoware, Jones, and Reinhart (1942). This method of expression, rather than the King-Armstrong phenol unit, was chosen to allow a ready comparison with the 5-nucleotidase activities.

Results

When determinations of the two enzyme activities were made on a number of different sera it became apparent that results fell into three main groups (Table I).

TABLE I

SERUM ADENOSINE-5-PHOSPHATASE AT *p*H 7.4 COMPARED WITH NON-SPECIFIC GLYCEROPHOSPHATASE AT *p*H 9.3 ("BODANSKY UNITS")

Group	Adenosine-5-phosphatase at *p*H 7·4	Alkaline Phosphatase at *p*H 9·3	Disease State (No. of Cases Examined in Brackets)
I	Normal 0–1·6 units	Normal 2–5 units	Rheumatoid arthritis (17). Tuberculous spine (12). Pulmonary tuberculosis (1). Osteoporosis (1). Myeloma (2). Osteogenic sarcoma. hip (1). Swollen ankles (1). Gouty toes (1). Kyphosis (2). Muscle injury (1). Osteitis, Osteoarthritis, Iliocondensans (1). Fragilitas ossium (1). Nephrocalcinosis (1). Suspected jaundice (13). Xanthomatosis (1). ? Myxoedema (1). Prolapsed disc (1)
II	Normal 0–1·6 units	Raised 9–19·3 units	Paget's disease (3). Rickets (6). Albright's syndrome (1). Breast cancer (2). Spinal neoplasm (1)
III	Raised 1·7–36 units	Raised 4·1–22 units	Tuberculosis with amyloid (4). Jaundice (22). Polycystic kidney with uraemia (1)

First Group.—Both 5-nucleotidase and non-specific alkaline phosphatase values were low in normal men, and a number of miscellaneous conditions.

Second Group.—5-Nucleotidase was low and non-specific alkaline phosphatase values were high in rickets, Paget's disease, osteogenic tumours, Albright's syndrome, breast cancer, and spinal neoplasm.

Third Group.—Both 5-nucleotidase and non-specific alkaline phosphatase values were high in obstructive and infective jaundice, liver haemangioma, lung or bone tuberculosis with amyloid, and polycystic kidney.

Results of other criteria for liver function have not shown precise correlation with the high nucleotidase values in the third group as can be seen in Table II. Sera with low bilirubin contents may have high nucleotidase activities and raised nucleotidase does not exactly parallel raised serum alkaline phosphatase. Thus one case (M. H.) showed a low 5-nucleotidase (1.5) value during the peak of his "jaundice" (bilirubin 17 mg./100 ml. and 9.9 units alkaline phosphatase) but two months later a higher 5-nucleotidase (5.7) value with lowered alkaline phosphatase (4.1) and no bilirubinaemia. In general, however, it can be said that serum cholesterol, alkaline phosphatase, and 5-nucleotidase are increased together but not to the same extent.

TABLE II

DETAILS OF CASES IN GROUP III

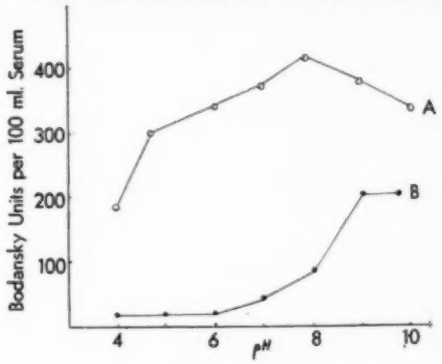
Name	Condition	Adenosine Phosphatase "Units"	Glycerophosphatase (Bodansky Units/100 ml.)	Bilirubin (mg./100 ml.)	Serum Cholesterol (mg./100 ml.)
B.N.	Tuberculous spine with amyloid	6·6	10·4	0	—
M.H.	" " "	5·7	4·1	0	—
R.S.	Pulmonary tuberculosis with amyloid	6·0	19·2	0	—
J.F.	" " "	14	13·8	0	—
5039	Polycystic kidney ..	5·6	20·0	0·6	—
W.F.	Infective hepatitis ..	2·1	12·3	5·9	221
1644	Biliary cirrhosis ..	35·5	18·4	14·7	427
P.O.	Obstructive jaundice ..	3·6	9·9	+	—
875	" " ..	13·9	13·9	8·3	450
5285	" " ..	1·7	12·3	30·5	131
953	" " ..	27·4	33·3	18·0	600
5241	" " ..	16·0	23	2·3	302
5450	" " ..	6·9	13·7	32	70
5429	" " ..	5·3	9·6	30	128
5408	" " ..	4·0	8·8	25	75
E.K.	" " ..	4·5	11·5	+	—
S.	" " ..	8·0	17·6	7·3	—
H.H.	" " ..	4·4	11·4	10·4	255
T.	" " ..	4·6	11·8	4·9	216
N.	Carcinoma of bile duct with obstruction	8·1	25·7	4	—
J.B.	Jaundice, obstructive	4·7	22·5	5·8	640
M.N.	" "	7·4	15·4	19·7	234
F.M.	Gallstones	20	23	15	515
C.B.	Chronic cholecystitis	20·3	22·2	7	375
R.U.	Chronic cholangitis ..	15·7	16·5	19	578
Pr.	Carcinoma of bronchus + jaundice	36	22	15	—
M.M.	Haemangioma of liver probably with obstruction	10·5	20·7	6·6	490

The serum activity curves at varying *p*H values from a particular case of jaundice having high 5-nucleotidase activity are shown in Fig. 1 with adenosine-5-phosphate and β-glycerophosphate as substrates. This gives further proof of the occurrence of high concentrations of this specific 5-nucleotidase with an optimum at about *p*H 7.5 in some sera.

Attempts to induce liver damage in two rabbits by twice weekly carbon tetrachloride (1 ml./kg.) injections for six weeks, although raising the serum cholesterol from 23 to 297 mg./100 ml., did not alter the 5-nucleotidase from 0.2 units although the alkaline phosphatase increased slightly from 0.5 and 0.9 units to 0.85 and 1.9 units per 100 ml. respectively.

Similarly in attempts to produce liver disease in rats, 5% by weight of bromobenzene was added to

FIG. 1.—Hydrolysis of adenosine-5-phosphate and of β-glycerophosphate by blood serum of jaundiced patient (Pr.) at varying *p*H values. A = adenosine-5-phosphate; B = β-glycerophosphate.



the stock diet of 150 g. rats for 6 weeks as described by Koch-Weser, de la Huerga, Yesinick, and Popper (1953). The serum 5-nucleotidase and alkaline phosphatase of these rats, respectively 2.1 and 14.3, was not appreciably different from the 2.1 and 14.6 of the control group.

Discussion

Although intestine and liver are regarded as contributors to the normal serum alkaline phosphatase level there seems general agreement that bone also is the source of at least some of it, and that the hyperactive osteoid tissue in, for example, rickets, produces the high level in that condition. Serum alkaline phosphatase is elevated only in cases where formation of some kind of osseous tissue is proceeding. In hyperparathyroidism, Paget's disease, and rickets, serum alkaline phosphatase values run roughly parallel with the severity. Normal values are found in osteoporosis and in bone tumours unless they are osteoplastic.

The results described above show that a high serum alkaline phosphatase level from liver disease is usually accompanied by a 5-nucleotidase value increased as much as a hundredfold whereas that from bone disease is not. Bone, therefore, is not likely to be the source of serum 5-nucleotidase.

The authors wish to express grateful thanks to Professors C. H. Gray, E. J. King, and N. F. MacLagan and Dr. D. N. Baron and Miss E. W. M. Johnson for making available some of the sera and information concerning them.

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THE ESTIMATION OF BUTAZOLIDIN IN BLOOD

BY

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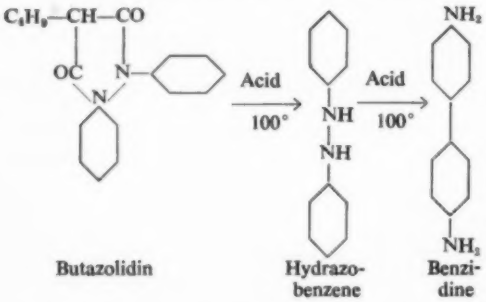
Butazolidin, or 3,5-dioxo-1,2-diphenyl-4-n-butylpyrazolidine, has recently been given extensive trials in the treatment of rheumatoid arthritis. Estimations of drugs in blood are often of some interest during the early period of their use, as it may be found possible to relate both the therapeutic response and the toxicity (if any) to the blood concentration. Toxicity, for example, may be related to a cumulative effect of the drug as is the case in bromide therapy.

Butazolidin was first estimated in blood by Pulver (1950), who hydrolysed the substance, with formation of hydrazobenzene, which then rearranged to benzidine. This latter substance was estimated colorimetrically by a diazo reaction. Brodie and Burns (1952) extracted acidified plasma with heptane, and, after returning the butazolidin to aqueous alkali, determined the optical density at 265 mμ with an ultra-violet spectrophotometer. The method was modified by Stanfield, Brodie, and Yeoman (1953), and it is simple and accurate. It requires, however, the use of an expensive instrument which is not generally available. The present method was based on that of Pulver (1950) and makes use of the following chain of reactions:



Butazolidin — Hydrazobenzene — Benzidine

Preliminary experiments on normal serum, fortified by the addition of "butazolidin," showed that considerable losses of the drug occurred on protein precipitation by the commonly used reagents. Using trichloracetic acid, the recovery was 31%. A mixture of zinc sulphate and sodium hydroxide, as used by King (1946), gave a recovery of 45%. These losses are probably to be explained by a strong binding of "butazolidin" to plasma proteins, which has been reported by Burns (1953). Extraction of blood or serum by organic solvents, however, gives good recoveries. Acetone was preferred in the present method, but its use requires precautions against loss of solvent by evaporation.

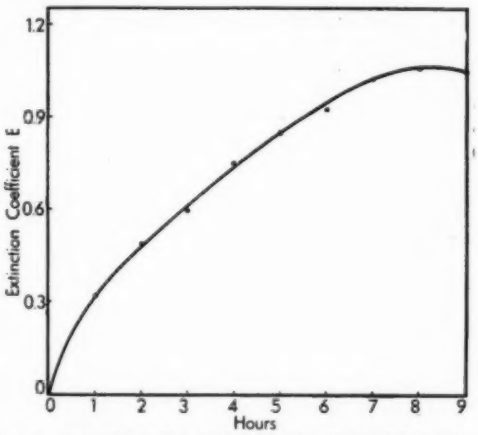


FIG. 1.—Rate of formation of benzidine from "butazolidin" under the conditions of hydrolysis used in the estimation.

Pulver used somewhat drastic hydrolysis conditions for the conversion of "butazolidin" to benzidine, involving heating to dryness with *p*-toluenesulphonic acid. Hydrolysis by dilute sulphuric acid, however, has been substituted in the present technique, as it was found to give more reproducible results. Fig. 1 shows the formation of benzidine plotted against hydrolysis time under these conditions. The maximum yield of benzidine was obtained after eight hours' hydrolysis, and corresponded to 83% of the stoichiometric yield. After four hours' hydrolysis the yield was 58% of the theoretical.

The benzidine produced was estimated by diazotization, followed by coupling with N-sulphatoethyl-m-toluidine, this reagent being preferred to the N-N-dimethyl-a-naphthylamine used by Pulver.

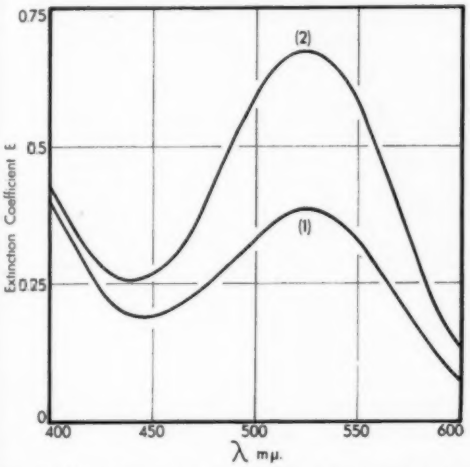


FIG. 2.—Absorption spectra of the purple pigments derived from (1) the "butazolidin" standard used in the estimation; (2) substitution of the theoretical maximum amount of benzidine for the "butazolidin" standard.

The absorption spectrum of the purple pigment formed in the present method is shown in Fig. 2. The wavelength of maximum light absorption was 525 mμ and was the same for both the colour derived from a solution of benzidine and from hydrolysed "butazolidin." A linear relation was found between optical extinction, measured at 525 mμ, and concentration of "butazolidin," up to a concentration equivalent to a serum level of 25 mg./100 ml. Normal serum gives a negligible colour by the method. Recoveries of "butazolidin" added to normal serum are shown in Table I.

TABLE I
RECOVERIES OF BUTAZOLIDIN ADDED TO 1 ML. SERUM

Added (μg.)	Found (μg.)	Recovery (%)
50	43	86
100	89	89
150	136	91
200	190	95
250	236	95

Method

Reagents.—The following are required :—

(1) A.R. acetone.

(2) Normal sulphuric acid.

(3) 0.5% Sodium nitrite, which is stored at 0° C.

(4) 1.8% N-Sulphatoethyl-m-toluidine* in water, stored at 0° C., keeps for several weeks, but should be discarded if a brown tint appears.

(5) Standard "butazolidin" solution, 3.0 mg.% in A.R. acetone, which should be stored in a tightly stoppered bottle.

Procedure

Serum, 1 ml., is added to 4 ml. acetone in a glass-stoppered centrifuge tube of 8 ml. capacity. After shaking well, the mixture is stood for 30 minutes and is then centrifuged, the stopper being retained in the tube during this procedure. Duplicate portions of 1 ml. of supernatant solution are removed and transferred to conical pyrex centrifuge tubes of 1 ml. capacity graduated in tenths of a ml. A duplicate standard is set up simultaneously by taking two portions of 1 ml. of standard "butazolidin" solution in similar graduated centrifuge tubes.

The bulk of the acetone is now cautiously evaporated off in a hot-water bath, the tubes being agitated gently to prevent loss by spurting or bumping. N sulphuric acid, 1 ml., is added to each tube, and the tubes are placed in a boiling-water bath for four hours. At half-hourly intervals the volumes in the tubes are restored to 1 ml. by the addition of distilled water. The final volume is 1 ml. After hydrolysis the tubes are chilled in ice-water, and 0.5 ml. of ice-cold sodium nitrite solution, followed by 2 ml. of ice-cold N-sulphatoethyl-m-toluidine, is added, the solution being mixed well after each addition. The purple colours are allowed to develop for 30 minutes at room temperature. The serum tubes have a slight turbidity at this stage due to the presence of unsaponified lipoid material. This turbidity is removed by adding 0.2 ml. of 40 to 60° light petroleum, shaking briefly, and centrifuging. Then 3 ml. of the aqueous phase is removed. The standards are similarly treated with light petroleum. The coloured solutions are read in a spectrophotometer at 525 mμ, or in a colorimeter with appropriate filter. The standards correspond to a serum level of 15 mg./100 ml.

The reproducibility of the method is rather poor, due to slight differences in the conditions of hydrolysis. In a series of 50 estimations carried out in duplicate on sera of patients receiving the drug the paired results differed from their individual means by amounts varying from ±0.0 to ±0.8 mg./100 ml., the average difference being ±0.3 mg./100 ml. For a serum level of 10 mg./100 ml. this would correspond to an error of ±3%.

Estimations of "butazolidin" in blood, plasma, or serum have been reported by Pulver (1950), Gsell and Müller (1950), Domenjoz (1952), Burns, Schulert, Chenkin, Goldman, and Brodie (1952), Currie, Peebles Brown, and Will (1953), Burns, Rose, Chenkin, Goldman, Schulert, and Brodie (1953), Meanock (1953), Stanfield *et al.* (1953),

* Supplied by Imperial Chemical (Pharmaceuticals), Ltd., Manchester.

Yü, Sirota, and Gutman (1953), and Pemberton (1954). As some of these analyses were carried out on whole blood and some on plasma or serum,

TABLE II
DISTRIBUTION OF BUTAZOLIDIN BETWEEN RED CELLS AND PLASMA

Case No.	Haematocrit	Plasma Level (mg.%)	Whole Blood Level (mg.%)	Concentration Calculated in Red Cells (mg.%)
1	36	4·5	3·0	0·5
2	30	6·5	4·5	0·0
3	37	7·5	4·5	−0·5
4	36	8·0	5·0	−0·5
5	43	11·0	6·5	0·5
6	40	15·5	10·0	1·5

it was considered desirable to examine the distribution of the drug between the red cells and the plasma. Specimens of heparinized blood were collected from patients receiving "butazolidin," and simultaneous estimations were carried out on whole blood and plasma, the haematocrit being determined on a separate specimen. The concentration of "butazolidin" in the red cells was then calculated from the equation:

$$x = \frac{100b - 100p - ph}{h}$$

where b, p, and x are concentrations of "butazolidin" (in mg.%) in whole blood, plasma, and red cells respectively, and h is the haematocrit. The results are shown in Table II. The drug was found to be absent or present only in traces in the red cells.

Discussion

There appears to be general agreement that the blood concentration of "butazolidin" is related to its therapeutic effect. Currie *et al.* (1953) considered a plasma level of 8 to 11 mg.% to be required for the suppression of symptoms in rheumatoid arthritis, and Burns *et al.* (1952) say "about 10 mg.%." Meanock (1953) considered the therapeutic range to be 5–10 mg.% in whole blood, and stated that levels in excess of these did not give an additional response. Pemberton (1954), whose analyses were carried out on serum, considered levels of 12 to 16 mg.% to be desirable. A plasma level of about 10 mg.% appears to be of some physiological significance in the treatment of gout, as Yü *et al.* found uricosuria to be induced at this level. The drug is not cumulative, and Burns, Rose, Chenkin, Goldman, Schulert, and Brodie (1953) found a ceiling plasma level to exist for a given person. The present investigations confirmed this; the highest serum level of a series of about 300 estimations was 22.5 mg.%. The serum or plasma level is not proportional to the dosage and increases by a proportionately smaller amount as the dosage is increased. For a given daily dosage, however, the serum level is generally quite constant over a considerable period of time. After administration has been stopped the "butazolidin" is slowly eliminated from the body, and may still be detected in the serum three weeks later. These points are illustrated by Fig. 3, which shows daily estimations on two patients with rheumatoid arthritis.

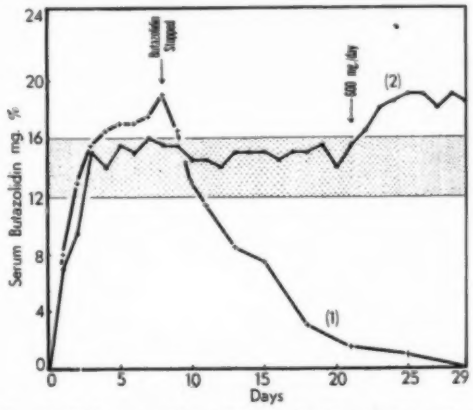


FIG. 3.—Daily serum "butazolidin" estimations on two patients with rheumatoid arthritis. Patient (1) received 800 mg. per day until the eighth day, when a toxic reaction occurred, and the drug was discontinued. Patient (2) received 400 mg. per day until the twenty-first day and 600 mg. per day after that.

Complete and rapid absorption of "butazolidin" from the alimentary tract has been reported by Currie *et al.* (1953) and by Burns *et al.* (1952). We were not able to reproduce these findings, however, and after administration of a single dose of 400 mg. of the substance in the form of enteric-coated tablets to two patients with rheumatoid arthritis, maximum serum levels of less than 1 mg.% were found. Stanfield *et al.* (1953) and others have described the finding of such tablets intact in the stools. In addition to the possibility of incomplete absorption, the rate of metabolism of "butazolidin" varies greatly from patient to patient, and the serum level is not generally predictable from a consideration of the dosage schedule. Serum "butazolidin" estimations are therefore of some value in those patients who fail to give an adequate response to the drug. If the serum level is found to be within the required therapeutic range administration of "butazolidin" can be discontinued as being ineffective. If, however, the

serum level is undesirably low, an increased dosage can be considered.

No data relating the blood level of "butazolidin" with its various toxic manifestations have yet been published, though Meanock (1953) considered such a relationship to exist. Of eight patients in the present series who reached serum levels of over 20 mg.%, only one developed what was considered to be a toxic reaction. This was of a mild character (sore throat and excess salivation). Of six patients who developed gastro-intestinal disturbances, and whose serum "butazolidin" level was known at the time of the reaction, two had levels below the range 12–16 mg.%, two in that range, and two slightly above (both 17.5 mg.%). This limited number of cases, therefore, does not suggest that the estimation is of value in the avoidance of toxic reactions.

I am much indebted to Dr. H. S. Barber and Dr. Lois Stent, in cooperation with whom the work was carried out. A specimen of purified "butazolidin" was supplied by Geigy, Ltd.

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J. clin. Path. (1954), 7, 348.

TECHNICAL METHODS

AN INTERCHANGEABLE THROTTLING DEVICE FOR PIPETTES

BY

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Many techniques in immunology require the rapid and accurate measurement of a large number of small volumes of fluid. A day's work in a routine Wassermann laboratory doing 100 tests, for instance, involves the performance of nearly 1,500 volumetric measurements.

Of the many devices for performing such measurements, one of the simplest and most satisfactory is the throttled pipette shown in Fig. 1*a*. It is a slightly modified version of one described by Wright and Colebrook in 1912, and has been in continuous use in the laboratories of St. Mary's Hospital and the Wright-Fleming Institute for many years. It consists of two pieces of thin-walled glass tubing, one, the barrel, having an internal diameter of about 6 mm., and the other, the stem, which is graduated, having an internal diameter of about 2 mm. The stem is fitted into the barrel with sealing-wax so as to make an air-tight junction. The throttle, a finely drawn out piece of capillary tubing, is fitted into the open end of the barrel with a further air-tight plug of sealing-wax as shown in the figure. A rubber teat is then placed over the throttled end of the barrel. When a fine throttle is used expansion of the compressed teat is so slow that the volume of fluid in the graduated stem can be very accurately controlled, and small volumes of, for example, 0.1 ml. of fluid can be discharged into a series of tubes with considerable speed and accuracy. This design of pipette has three major drawbacks. First, the sealing-wax joints are liable to fracture; second, the pipette cannot be cleaned without melting the joints and subsequently reassembling the apparatus; and, third, the throttle tends to become blocked by minute particles of dust.

The pipette described below (Fig. 1*b*, *c*, and *d*) has been designed to overcome these disadvantages. It is made entirely of borosilicate glass. The stem usually consists of a standard 1.0 ml. "blow out" pipette calibrated in 0.05 or 0.01 ml. divisions (Fig. 1*b*). Alternatively, a 0.2 ml. "blow out" pipette graduated in 0.05 ml. divisions will also be found useful. The pipette is fused to the "male" component of an interchangeable

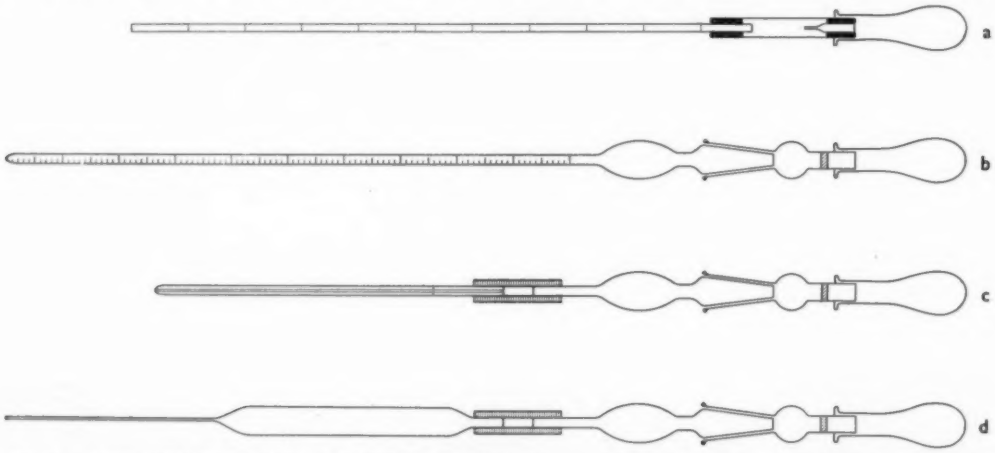

FIG. 1*a*.—Almroth Wright throttled pipette. The joints are made with sealing-wax. *b*. Throttled pipette with ground glass joint. The stem is a standard 1.0 ml. "blow out" pipette graduated in 1/100 ml. divisions. The pipette, joint, and throttle are made of borosilicate glass. *c*. Throttle connected to a 0.1 ml. capillary pipette through a short piece of pressure tubing. *d*. Throttle connected to a finely drawn Pasteur pipette (see text).

conical ground glass joint No. B.7 and a bulb of 1–2 ml. capacity is blown between the two. This acts as a reservoir for any excess fluid accidentally taken up, and so minimizes the danger of aspirating fluid into the throttle.

The throttle is made by fusing a sintered glass disc 10.0 mm. in diameter into the glass tubing immediately above the "female" component of the ground glass joint. Sintered glass of porosity 2 or 3 has been found satisfactory. During the process of fusing in the disc, a certain depth of the sintered glass will become solid, leaving only a central porous area. By increasing the amount of heating, this area can be reduced with a consequent increase in the throttling effect. In this way throttles of different speeds can readily be produced.

The "male" and "female" components are now joined together and a rubber teat is fitted over the open end of the throttled "female" component. The ground glass joints fit so well that they will not come apart, even after hours of use. Some may prefer to use spring clips attached to glass hooks on the two sides of the joint, but these have not been found necessary, and add to the weight of the pipette, an important point when very large numbers of measurements are being made.

If a 1.0 ml. pipette having an unconstricted tip is attached to the throttles then these can be graded according to the time taken to fill the pipette with water. Throttles giving filling times of about four to five and about 15 seconds have been found to be the most useful. These, of course, give somewhat greater slowing when attached to pipettes having normally constricted tips.

This design of pipette has several advantages: (1) The pipette can readily be separated from the throttle for cleaning, or a new pipette can be substituted. (2) The throttle can be instantly changed for one which is faster or slower. (3) The pipette and throttle can be sterilized by heating, as they are made entirely of borosilicate glass. (4) The pipette can be used for the accurate measurement of small volumes of fluid when oral suction is undesirable. (5) The use of the pipette can be widely extended as shown in Fig. 1*c* and *d*, if one "male" component is cut off a short distance below the joint and a piece of rubber pressure tubing is attached. A large variety of small pipettes can be inserted into the open end of the rubber tubing, and so be connected to the throttle. (6) If a finely drawn out Pasteur pipette is attached to a slow throttle, the apparatus will be found to be extremely useful for aspirating one layer of fluid from above another, without disturbing the boundary. This has been found particularly valuable when isolating protein fractions separated in a small Tiselius type of electrophoresis apparatus.

Accuracy of the Throttled Pipette

The accuracy of the throttled pipette, when used to make a series of small volumetric measurements, was compared with that of the same pipette used in the normal way, the outflow being controlled by keeping a finger over the open end of the "male" component, the throttle having been detached. Water, 10.0 ml., was measured accurately into each of a series of 24 test tubes. Then 0.1 ml. of a solution of methylene blue in water was measured into 12 of these tubes with a throttled, graduated 1.0 ml. pipette and into the 12 remaining tubes with the pipette used in the normal way. The tubes were closed with rubber bungs and the contents were mixed by inversion. In each experiment, the pipette was filled to the 1.0 ml. mark, and 0.1 ml. of the methylene blue solution was run into each tube until the pipette was empty. It was then refilled, and 0.1 ml. of the solution was run into the two remaining tubes. The coefficient of extinction of each sample was then estimated with a Spekker photoelectric absorptiometer. The concentration of methylene blue used in these experiments was adjusted so that the final solution, after dilution, had a coefficient of extinction of approximately 0.3, as this is in the region of maximal sensitivity of the absorptiometer. The results with the pipette used in the normal way and with the throttle are shown in Table I. The means of the coefficients of extinction are identical with the two methods, and there is no significant difference between the standard deviations and the coefficients of variation, what slight difference there is being in favour of the pipette used with the throttle (σ with throttle=0.0041, V=1.44; σ with pipette used normally=0.0055, V=1.93). This test was performed to investigate the use of the pipette in producing accurate dilutions, rather than by weighing volumes of water discharged, as this is the way in which the pipette will generally be used. These results show that the use of the throttle involves no loss of accuracy in the measurement of small volumes of fluid.

TABLE I

COMPARISON OF ACCURACY OF DILUTIONS MADE WITH A THROTTLED PIPETTE AND WITH THE SAME PIPETTE USED IN THE NORMAL WAY

Tube No.	Dilution Performed with Throttled Pipette		Dilution Performed with Pipette Used Normally	
	Coefficient of Extinction		Coefficient of Extinction	
1	0·28		0·285	
2	0·2925		0·29	
3	0·285		0·285	
4	0·2825	Mean=0·28458	0·29	Mean=0·28458
5	0·28		0·2825	
6	0·285	σ=0·0041	0·285	σ=0·0055
7	0·285		0·285	
8	0·28	V=1·44	0·2825	V=1·93
9	0·2925		0·27	
10	0·2825		0·29	
11	0·285		0·29	
12	0·285		0·28	

The author is indebted to the Medical Research Council for a grant for expenses; and to Mr. William Brough for his help in making this pipette.

The pipette can be obtained from Messrs. Ebcol Scientific Glassware Co., Webbs Works, Chislehurst West, Kent.

REFERENCE

Wright, Sir A. E., and Colebrook, L. (1912). *Technique of the Teat and Capillary Glass Tube.* Constable, London.

J. clin. Path. (1954), 7, 350.

ANTIBIOTIC SENSITIVITY TESTS: A RAPID METHOD SUITABLE FOR MULTIPLE CULTURES

BY

J. G. P. HUTCHISON

From the Department of Bacteriology, Royal Victoria Infirmary, Newcastle-upon-Tyne

(RECEIVED FOR PUBLICATION FEBRUARY 24, 1954)

The apparatus described was designed to deal with a large batch of sensitivity tests with rapidity and accuracy. The method is suitable for routine laboratories where many such tests are demanded.

Method

An illuminated grid system is used for the orientation of cultures and of blood agar plates containing suitable antibiotic concentrations. A small drop of a broth culture is seeded on to each plate in a position defined on a duplicated paper facsimile of the grid.

Details of the Grid Construction.—Figs. 1–4 show the general design of the apparatus.

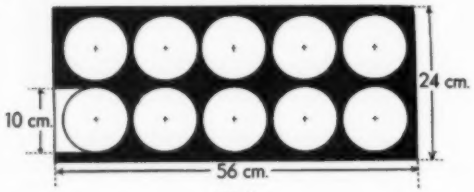

FIG. 1

A sheet of "perspex" 24 × 56 × 0.3 cm. forms the main table beneath which 10 opaque grids are stuck. The 10 grids of black x-ray backing paper are cut simultaneously round an inverted petri-dish lid (10 cm.) with a sharp knife. A circular template of white paper (10 cm. diameter) is ruled into 1.0 cm. squares (Fig. 2 lower half) and those for subsequent perforation marked (Fig. 2 upper half); all discs are then stapled together. With a gentle screwing motion, a very sharp cork borer, giving a hole 6 mm. in diameter, is driven through the disc pad in the centre of each marked square. On completion the staples are removed and the 10 identical black grids stuck to the "perspex" table. A very thin glue of perspex shavings in chloroform may be used, the grid being immersed in the glue and quickly transferred to its exact position; owing to rapid setting, no change is possible once the grid is in place. Subsequently transparent areas between the grids are coated with dead-black paint (Fig. 1 in black).

The completed table is mounted in a frame which pulls out from beneath the laboratory bench like a drawer (Fig. 3).

Illumination.—A 100-watt lamp with a metal reflector is fixed to the underside of the bench not less than 2 ft. from the grid system in a working position. An aluminium reflector (56 × 30 cm.) attached to the draw-out frame and inclined at about 45° changes the direction of light from the horizontal so that it shines vertically upward through each grid. The reflector is mounted on hinges and can be folded up against the underside of the grid table when not in use.

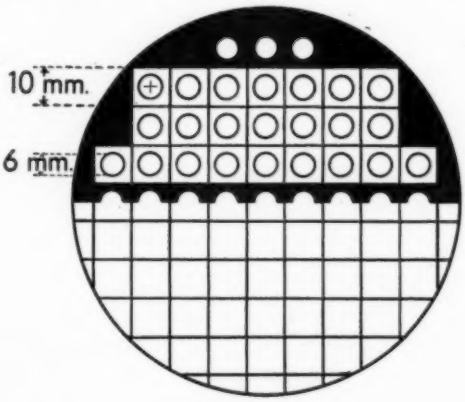

FIG. 2

Pipettes.—Pasteur pipettes are drawn from 5 mm. glass tubing into fine pliable points. The exact relationship between end-bore and flexibility is a matter of experience, but they should allow about 0.05 ml. of broth to be drawn up in a matter of seconds: if too fine filling is slow, if too rigid inoculation of the plates is made difficult, since they may stick in the agar and become choked or deliver too large a drop.

Sterile pipettes are stored in aluminium cans. After use each is flushed with distilled water, boiled, and resterilized at 160° C.

Identification.—For identification, culture numbers are written down on forms (prepared on a duplicator) bearing a squared facsimile of the grid (Fig. 2 lower half).

Antibiotics.—Antibiotics are incorporated at suitable concentrations in a blood agar medium. For routine

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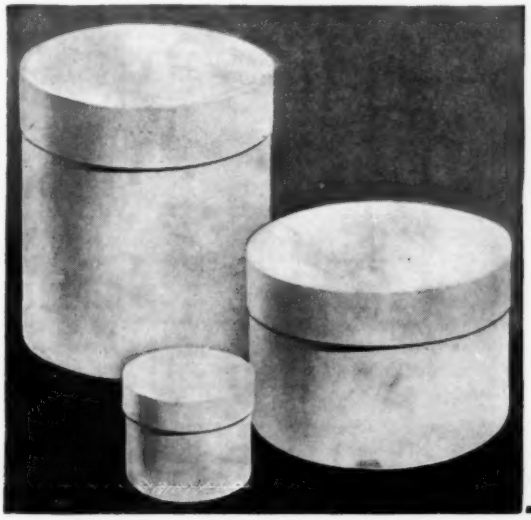

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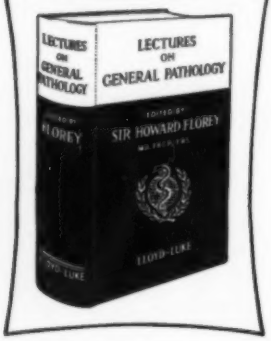

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LECTURES ON GENERAL PATHOLOGY

EDITED BY

SIR HOWARD FLOREY

M.D., F.R.C.P., F.R.S.

THIS book is based on the lectures given to medical students at the Sir William Dunn School of Pathology, Oxford. It is designed to give a background to modern work and to indicate some of the conclusions that have been reached by use of experimental methods in the study of disease. Special attention is given to the effect of various kinds of injury. For example, the course of inflammatory and immune reactions to infection are dealt with at length, and the special characteristics of injury due to trauma and to radiation are also considered.

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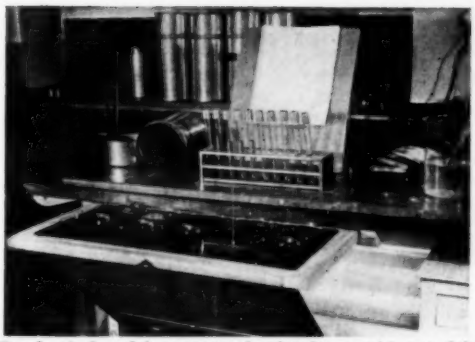

FIG. 3.—A view of the apparatus showing the general layout of the grid table and reflector together with accessories.

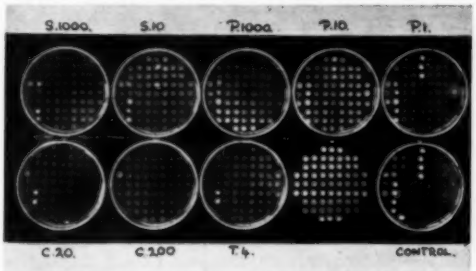

FIG. 4.—The grid table with plates laid out as for inoculation. One plate has been removed to show the grid; the remainder have been inoculated with a few cultures and incubated to show growth. The plates in this case contain: penicillin 1.0, 10, and 1,000 units per ml.; streptomycin 10 and 1,000 μg. per ml.; chloromycetin 20 and 200 μg. per ml.; and terramycin 4 μg. per ml.

purposes penicillin (at three levels), streptomycin, chloromycetin, and terramycin (two levels each) are used, a fresh batch of plates (10 cm. diameter) being poured weekly.

Test-tubes.—Test-tubes with "oxoid" aluminium caps containing broth or peptone water are seeded and incubated for four hours at 37° for inoculation.

Procedure

Each petri dish is first marked across its diameter with a line (in black) bearing a cross at one end. This diameter, when a plate is in the correct position, falls north–south down the centre of the grid over which it is placed, with the cross to north. The plates in position, a pipette is charged with about 0.05 ml. of the first broth culture and a 3–6 mm. spot touched off on to each agar surface in turn. Subsequent cultures follow in the same manner so that in any one series of plates a given specimen occupies an identical position on each. It is found easier to inoculate successive cultures in a north to south direction. After drying the plates are incubated.

Reading is done most easily by taking one plate at a time and noting all reactions in a suitable book. Since it is the crux of the method that all cultures and their corresponding numbers must follow an invariable sequence the duplicate form need rarely be consulted.

Speed of performance may be judged from the fact that after some practice the inoculation time of 10 plates for 60 cultures is about 30 minutes and reading time 20 minutes.

The method has been in routine use in this department for a year and has proved satisfactory.

My thanks are due to Dr. C. A. Green of this department for valuable help in connexion with this paper.

J. clin. Path. (1954), **7**, 352.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 53rd GENERAL MEETING

The 53rd general meeting was held in Brighton from September 30 to October 2, 1954. Papers were read at seven sessions, and demonstrations occupied one. Summaries of some of the papers are printed below.

Fibroelastosis of the Foetal Heart

J. F. HORLEY (Brighton) described three examples of fibroelastosis. All were in babies born to healthy mothers who had suffered no illness during pregnancy. Post-mortem examination was unremarkable except for the changes in the heart. There was striking thickening of the endocardial layers of the left ventricle in each case. The weight of the heart varied from 17 to 25 g. All three showed small left ventricles, two extremely small. In one there was complete aortic stenosis and in the other two partial fusion of aortic cusps without gross scarring.

Evidence was produced to suggest that within the widening concept of fibroelastosis a small group consisting of a hypoplastic left ventricle, a thickened endocardium, and degrees of aortic cusp fusion could usefully be separated. In this group the most likely explanation of the thickened endocardium was fibrous replacement of ischaemic myocardium.

Clinico-pathological Aspects of Non-specific Oesophagitis

K. V. LODGE (Manchester) said that his paper was based on a macroscopic and microscopic study of the oesophagi from 500 unselected hospital necropsies, in which the overall incidence of oesophagitis was found to be 36%. A similar examination on 100 cases of "sudden death" showed an incidence of only 8%. Analysis of the 500 cases in relation to primary disease, symptoms, and treatment suggested reflux of acid contents from the stomach as the main cause of the lesion. Vomiting and gastric intubation are thus contributory factors, but in the majority of cases the horizontal posture of the recumbent patient is of most significance in increasing regurgitation and stasis of gastric contents in the lower oesophagus.

The importance of reflux was confirmed by experiments in the rat. The lesions found in man are reproduced by a diet lowering the *p*H of the gastric contents combined with an operation to relax the proventriculo-oesophageal junction.

The clinical importance of oesophagitis as a cause of stricture and shortening of the oesophagus was stressed. It was shown that 20.5% of the lesions in this investigation were of sufficient severity for such fibrosis to have resulted had the patients survived.

Pathology of a Chronic Infection with Salm. Typhimurium

G. R. OSBORN (Derby) first demonstrated gall-bladder lesions in a carrier of paratyphoid B who had remained free of the organisms after cholecystectomy, and second liver biopsies and necropsy findings in a girl who died at the age of 5 after being infected with *Salmonella typhimurium* for over three years. A multilobular cirrhosis developed. Masses of typhimurium bacilli, proliferating or lying dormant with little reaction to them, were found in small bile ducts when the disease had been in progress for 11 months and again *post mortem*. In the early stages she responded to chloromycetin and aureomycin, but then became resistant. Later she appeared to recover after treatment with terramycin for one year. Three months after treatment with this drug was stopped she died unexpectedly with necrotic lesions of Peyer's patches resembling those of typhoid. Typhimurium bacilli were recovered easily from the liver, bile, and bowel; culture from the spleen was negative.

Theca Cell Tumours of the Ovary

R. I. K. ELLIOTT (Brighton) said that theca cell tumours of the ovary fell into two groups, the xanthofibromata, which resemble fibromata of the ovary but are characterized by yellow areas rich in lipoid, and the thecomata, which are difficult to distinguish from granulosa cell tumours. A reticulin meshwork in the thecoma, but not in the granulosa cell tumour, is of some value in differentiation, but the occurrence of mixed tumours makes the boundary difficult to define.

Clinically the two types do not differ materially; both show endocrine effects in 60 to 70% of cases. The low rate of malignancy (3%) and the frequency of association with carcinoma of the endometrium (20%) are the other important features of this tumour group.

Sensitivity of M. tuberculosis to Anti-tuberculous Substances

LILIAN V. REILLY (Belfast) spoke of the sensitivity of *M. tuberculosis* to streptomycin, P.A.S., and isoniazid (I.N.A.H.). The most practical method of testing was found to be primary isolation of the strain on standard Loewenstein-Jensen medium and then subculture on the same medium to which had been added streptomycin, P.A.S., or I.N.A.H. More consistent results were obtained if completely fresh medium were used. The importance of combining two or more antituberculous substances in treatment to prevent the emergence of resistant strains was demonstrated. A combination of two substances, if the patient were already resistant to one of them, was ineffective. Fives cases of tuberculous meningitis had been infected with resistant strains.

Acquired Hypofibrinogenaemia

H. LEMPERT (Manchester) dealt with a case of carcinoma of the prostate with bone metastases in a man of 60 years. There were two severe haemorrhagic episodes occurring at an interval of eight months. In the first episode the fibrinogen of the plasma was reduced to 43 mg.% and the platelets to 50,000 per c.mm. The fibrinogen level returned to normal within five days, while the platelets took a few days longer. In the second episode, which ended fatally, the platelets were affected to a greater extent than the fibrinogen. Before death the platelets were 27,000 per c.mm. and the fibrinogen 120 mg.%. A fibrinolytic mechanism was demonstrated in the second attack. This confirmed the findings of Tagnon and his colleagues.[1]

In the case discussed necropsy showed no metastases in the liver.

Evidence appears good that the haemorrhagic state is brought about by factors released intermittently from the metastatic deposits in the bone marrow. These factors appear capable of destroying fibrinogen, fibrin, prothrombin, factor V, and platelets.

REFERENCE
[1]Tagnon, H. J., Schulman, P., and Whitmore, W. F. (1953). *Amer. J. med.*, **15**, 875.

Non-syphilitic Cold Haemoglobinuria without Raynaud's Phenomena or Cold Agglutinins

P. N. COLEMAN (Northallerton) described how, following a bathe in a cold sea, a schoolgirl aged 12 years collapsed with a violent shivering attack. Within a few days she was found to have haemoglobinuria. Previously she had enjoyed good health and there was nothing in her history or on physical examination to suggest congenital syphilis. At the time that the haemoglobinuria was noticed she also had a mild fever and a slight sore throat. Laboratory investigation showed Hb 75%, reticulocytes less than 1%, and an unmistakably positive Donath Landsteiner reaction to a titre of 1 in 32. Other tests were negative; these were Ham's test, tests for warm and cold agglutinins, and the direct and indirect Coombs tests.

All serological tests for syphilis, including the treponemal immobilization test, were negative. The mother's Wassermann and Kahn tests were negative. The mother was a blood donor who must have had a number of previous negative Wassermann tests. There was evidence of streptococcal infection. A throat swab gave a scant growth of *Streptococcus pyogenes* type 12 and the antistreptolysin titre of the patient's serum was raised to 500 units/ml.

After the initial attack haemoglobinuria did not return, but haemolysis continued during the next six weeks. Haemoglobin fell to 50% and there was now a reticulocytosis to 15%. The direct Coombs test became positive, but a satisfactory indirect test was never obtained. (It is possible that had the cells been sensitized in the presence of excess complement a positive indirect test might have been obtained.) The patient then appeared to make a complete recovery.

The case did not resemble the non-syphilitic cases of cold haemoglobinuria reported by Ferriman *et al.*[1] These cases were in elderly patients, in whom there were Raynaud's phenomena and high titre cold agglutinins; the Donath Landsteiner tests were negative or only feebly positive. The case had all the characteristics of the syphilitic cases yet was not due to this infection. It is possible that streptococcal infection was the cause.

REFERENCE
[1]Ferriman, D. G., Dacie, J. V., Keele, K. D., and Fullerton, J. M. (1951). *Quart. J. med.*, **20**, 275.

Hodgkin's Specific Findings in Bone-marrow Punctures

S. VARADI (Sheffield) said that in five cases of Hodgkin's disease (one previously reported in 1937) Reed-Sternberg cells were identified in the bone-marrow. He maintained that if marrow aspiration is performed at the site where the bone is tender on pressure the puncture is likely to display a pathological cytology. If enough material is available sections should also be prepared. If the marrow is fibrotic (three cases) the result of the puncture may be a nearly "dry tap." The cytological demonstration of Dorothy-Reed cells in the poor, hypocellular material in these cases will obviate the necessity of trephining. In some cases which histologically show a non-specific granulomatous lesion the cytological identification of Dorothy-Reed cells in the dry-fixed smear preparations will establish the diagnosis.

The Nature and Modes of Presentation of Myelofibrosis

M. S. R. HUTT, G. WETHERLEY-MEIN, and J. L. PINNIGER presented six selected cases of generalized marrow fibrosis. These cases showed that this condition may present with clinical and haematological features which differ from the classical picture of myelofibrosis. The blood pictures were respectively those of acute aleukaemic leukaemia, chronic myeloid leukaemia, subacute myeloid leukaemia, polycythaemia, and aplastic anaemia. It was emphasized that splenomegaly was not a constant feature and that the one common factor in all these conditions was the difficulty experienced in obtaining adequate marrow aspiration samples. Myelofibrosis should therefore be considered in any case of marrow dysfuncion associated with a "dry tap."

From a study of the histological material it could be recognized that this process was a primary proliferation of cells of the reticulum cell fibroblast series associated with varying degrees of proliferation of the haemopoietic cells. This association was most frequent with granulocytes, red cells, and megakaryocyte-like cells, and it was found that reticulin stains were of particular value in demonstrating it.

It was suggested that the clinical, haematological, and histological features of these cases established that myelofibrosis was a process related to, and of the same order as, the leukaemias and lymphomas.

Routine Production and Storage of Water of Extreme Purity (Conductivity Water)

G. DISCOMBE and R. F. JONES (Central Middlesex Hospital) described a convenient apparatus for preparing water purified by ion-exchange resins, together with a flashing conductivity indicator to show when the resin needed renewal. (To be published in full.)

J. clin. Path. (1954), 7, 354.

BOOK REVIEWS

The Physiopathology of Cancer. A Treatise for Investigators, Physicians and Students. Edited by F. Homburger and W. H. Fishman, with 28 contributors; Foreword by C. C. Little. (Pp. xviii+1,031; 152 illustrations. 135s.) London: Cassell & Co., Ltd. 1954.

Let me say at once how much I enjoyed browsing through this huge volume. Compiled by 28 contributors, each well qualified to write authoritatively on his selected branch of cancer research, the 1,031 pages are packed with many fascinating details about the histology, production, modifying influences, and spread of carcinoma of all sorts of animals from which are drawn illuminating analogies for human malignant disease. Each of the 32 chapters is generously supplied with references, which, so far as I can tell, were selected with discrimination and should prove of great benefit to the reader who wishes to extend his reading. Illustrations are clearly displayed and useful; apart from the lamentable muddle in the legends of Figs. 37, 38, and 39, they appear to have been chosen with care and they certainly supplement the text.

My task of reviewing this work within the narrow space available in the *Journal of Clinical Pathology* limits me to some remarks about the general plan of the undertaking and a brief description of one of the contributions. I hope in this way to induce the reader to look into the volume at his leisure. I feel sure he will not turn away disappointed, even though he encounters frustrations. The latter must be inevitable, not through any deficiency on the part of the writers but because of the many gaps in our knowledge of cancer that are displayed so courageously and grimly by the contributors.

Four approaches have been chosen for the attack on the cancer problem. Under " Biology " we find chapters devoted to experimental tumours of skin and subcutaneous tissue, alimentary tract, bone, brain, lungs, thyroid, breast, testis, endometrium, followed by discussions on the genetics of neoplastic disease, hormonal factors in carcinogenesis, virus-induced tumours, transplantable tumours, and nutrition and cancer. I have studied most of these chapters with some care, and I would like to congratulate the writers on the economy with which they have covered fields which are enormous and often confused.

Eight chapters are given up to chemistry and physics. Our own Alexander Haddow has contributed a fine monograph on " Chemical and Genetic Mechanisms of Carcinogenesis " which shows how much has come out of the Chester Beatty Research Institute in recent years. Nucleoproteins, enzymes, cytochemistry, and histochemistry as well as straightforward analysis are considered in relation to cancer tissue, and two chapters take up the subject of experimental cancer chemotherapy and radiation and cancer.

To " Clinical Investigation " are devoted four chapters wherein special attention is given to steroid metabolism in cancer, environmental and statistical studies. I confess that I found the section on clinical investigation in cancer research puzzling and sketchy. The last part of the book considers such topics as evaluation of diagnostic tests, chemotherapy, applied exfoliative cytology, radiation therapy, and isotopes.

I shall now tell you briefly how one of the chapters that I selected for more detailed study appealed to me In her discussion of the morphology of mammary tumours in mice, Dr. Thelma B. Dunn reminds us that these tumours were the early choice of investigators for serial transplantations and suggested the possibility of raising inbred strains of mice with a high and fairly predictable incidence of mammary tumours. From this came studies on environmental and extrinsic stimuli, quantitative work on tumour growth, attempts to find chemotherapeutic substances, and finally the recognition of the milk factor. The latter has since turned out to be a landmark for all sorts of cancer studies, many of which are still in their early stages. Even the histology of mammary cancer has had to be re-assessed in the light of milk factor influences, for it appears that the mammary tumour agent may exert a special stimulus on the acini so that tumours with a predominantly acinar structure develop at a relatively early age and cause death. More unusual forms of tumour emerge when the agent is absent and the mouse lives longer. Apparently tumours do not appear in the young unless the milk agent is present. Dr. Dunn illustrates these important points with excellent photographs, so that anyone who wishes to take up the study of experimental carcinogenesis in mice should find no difficulty in avoiding the many pitfalls that lie in wait for the unwary. It is unfortunate that the legends of Figs. 37–39 have been muddled as I pointed out above. And, finally, Dr. Dunn shows just how far the experimental results may be applied to human breast cancer. Both run through a pre-cancerous phase, often arise in multicentric foci, and develop a variety of histological pictures which grade into one another. But the human breast cancer is nearly always derived from the ducts, and alveolar cancer is rare, whereas in the mouse acinar or alveolar cancer is the most frequent type, especially in the presence of the milk factor. No convincing evidence for a tumour agent in human milk has yet been obtained. Data on the incidence of breast cancer in the relatives of cancer patients, and the records of women who never received human milk, do not support the opinion that a milk agent concerns human beings. Finally, to add to these difficulties we are told that the mouse mammary tumour agent may be transmitted by the male, and that many mouse tumours have no demonstrable tumour agent. One illuminating discovery deserves mention. It appears

that the elimination of one histological type of tumour characteristic of an early age period allows another type to develop at a later age. Does this mean that all our attempts to solve the cancer problem are doomed to failure?

In conclusion, allow me to make three final comments about the book. The clinical pathologist will find much useful information in chapter 24 on steroid hormone metabolism in cancerous subjects which he can ill afford to neglect in these days of adventurous surgery and hormone therapy. So, too, he cannot ignore the fine summary by Dr. Hueper of environmental, especially industrial, factors in human cancer. He will also discover that most of his enquiries are anticipated in the excellent accounts of diagnostic tests (chapter 28), radiation therapy (chapter 31), and isotopes (chapter 32). I hope that I shall be forgiven if I express my disappointment that serious consideration is not given to metastasis, which is not even mentioned in the index. And may I ask, What does "physiopathology of cancer" mean? I, for one, am no wiser after using this book, though I know a good deal more about the aetiology, pathogenesis, and investigation of cancer.

G. R. CAMERON.

Fundamentals of Neuropathology. By William Brooks Dublin. (Pp. 697; 786 illustrations. £6 13s. 6d.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1954.

Within recent years several books have appeared with the object of bringing neuropathology into line with general pathology. Dr. Dublin is to be congratulated on having succeeded in this endeavour, for the present weighty volume is an important and serious contribution to the subject of neuropathology: it is, however, based almost entirely on the American literature.

Neuropathology, explains the author, is but general pathology extended, and he sees no justification for the retention in our medical vocabulary of several terms which have grown up with this subject. Early on in this book occurs the statement, which will be regarded by many as heretical, that "Nissl stains, although traditionally important, contribute nothing." It is laudable to attempt the diagnosis of neuropathological conditions with paraffin sections stained with haematoxylin and eosin, but such statements as the above are sweeping in character and he would be a rash man who would deny the importance of celloidin preparation and the Nissl stain in many fields of neuropathology.

The various subjects are treated on conventional lines and the early chapters deal with the neuron, the glia, and their development. In the chapter on congenital abnormalities the author draws attention to the possible importance during the period of gestation of streptococcal pharyngitis in the mother and other forms of upper respiratory tract infection.

Inflammation is treated in a very broad way, for it includes such conditions as toxic encephalitis, diffuse sclerosis, and the Pelizaeus–Merzbacher disease. Disseminated sclerosis is classified as an "inflammation possibly allergic." The subject of allergic inflammation is well explained, particular attention being paid to the rheumatic group of diseases. He follows Rich (1946) in believing that the various disorders normally listed in this category represent varying types of reaction to different stimulating agents on a basis of allergic hypersensitivity. Cranial arteritis is regarded as a variant of polyarteritis nodosa. Dr. Dublin holds that both clinical, as well as pathological, distinction of primary from post-infectious encephalitis may be difficult or impossible owing to overlap of lesions of the two categories and to the appearance of combined forms, and that primary viral meningoencephalitis occurs more often than is generally realized.

The section on tuberculosis is comprehensive, for such "sub-varieties" as chronic granulomatous meningitis, which the author states may extend over a period of months or even one or two years, and meningoencephalitis *sine bacterium* are dealt with in detail.

The section on tumours is good, even if the presentation of the gliomas is unconventional and somewhat provocative. The familiar medulloblastoma, for instance, is listed as a neuroglioma grade 4.

But Dr. Dublin nobly grasps the bull by the horns when he includes arteriosclerosis and senile dementia among the metabolic disorders, although there are many who will regard the inclusion of Pick's disease within this category as premature. With some justification he holds that cerebral phlebosclerosis is of unappreciated frequency and importance.

In the chapter on injuries the author stresses the role of liberated enzymes, while the importance of fibrolytic degeneration of the dura (listed as a metabolic disease) in the aetiology of subdural haematoma is explained.

The technical section is good so far as it goes, but such standard methods as phosphotungstic acid, haematoxylin and Hortega's silver carbonate are not included, while the method of Holzer is not even mentioned.

This book has many illustrations, the majority of which are good. They are all well chosen and instructive. It is salutary, for instance, to be reminded that death because of cerebellar coning may and does follow lumbar puncture and that the orbital surface of the frontal lobe runs a risk of being seriously damaged during exploration of pituitary tumours.

W. MCMENEMEY.

An Introduction to Pathology. Second edition. By G. Payling Wright. (Pp. xii+636; 47 plates. 40s.) London: Longmans, Green. 1954.

When the first edition of Professor Payling Wright's *Introduction to Pathology* appeared in 1950 it was clear that it was in every sense a new book and no mere re-writing of the traditional texts. The book is based upon the author's undergraduate teaching course, but few even among senior pathologists will read it without both benefit and enjoyment. Although purporting to cover only the principles of the subject, the author makes such liberal use of examples that in fact many subjects are covered in considerable detail.

Professor Payling Wright brings a historical approach to his subject. With each of the main pathological processes sufficient account is given for the student to be able to picture how the modern views have evolved, and to see also the lines along which current research is progressing. Throughout the book emphasis is laid on functional pathology rather than on morbid appearances, and this is particularly notable in the nine chapters devoted to infection and inflammation. The whole book maintains a remarkable level of scholarship (especially for a one-man production), but the sections on infection, thrombosis, and neoplasia are perhaps deserving of special mention. A most useful feature to the more senior reader is a carefully selected bibliography conveniently placed as footnotes throughout the text.

In the new edition the whole text has been overhauled and references to recent works have been incorporated, but the main change is the addition of two new chapters on " Inflammation in Allergic Reactions " and " Tissue Reactions to Ionizing Radiations." These additions complete what is a quite outstanding exposition of the principles underlying present-day pathology and its application to the practice of medicine.

There may well be some who will feel that Professor Payling Wright has put too much into this book for the undergraduate, and that the introduction of so much historical and controversial matter may tend to obscure the more factual matter required for examination purposes. It is certainly true that the book's appeal will be more to the thoughtful student than to the one interested only in acquiring a minimum of facts for his examinations. To the postgraduate and to the young teacher of pathology it is an invaluable asset. It constitutes a most notable addition to the British literature on pathology.

T. CRAWFORD.

Practical Clinical Biochemistry. By Harold Varley. (Pp. viii+551; 70 figures. 42s.) London: William Heinemann. 1954.

The author's aim in writing this book is not clear. In the first paragraph of the preface he describes the book as a survey of the whole field of clinical biochemistry from the standpoint of workers in hospital laboratories. In the next paragraph we are told that the purpose of the book is essentially practical, but that it was felt that summaries of the findings in health and disease would add considerably to its value. If " clinical biochemistry " means anything, it means the application of biochemistry to the investigation and treatment of the patient; a survey of the subject demands more than summaries of the results of various tests in health and disease. Chemical pathologists and hospital biochemists will find this book valuable as a work of reference on technical matters. It is less suitable for use as a textbook.

Major criticisms are few. In the discussion of albuminuria in pregnancy the reader is left to infer that mild albuminuria in pregnancy is unimportant. There is no general discussion of error in the laboratory and little attempt is made to consider the accuracy of the various methods described.

Many minor matters invite comment. Some names are mis-spelt. The description of the breakdown of bile-pigments in the bowel is over-simplified and, since chemical formulae are quoted, incorrectly set out. It is to be regretted that the Biochemical Society's abbreviation for milli-equivalents (m.-equiv.) is not used. The work of Cole and Lathe on the van den Bergh reaction deserves mention. The steroids are only referred to by trivial names although the adoption of a systematic nomenclature (e.g. Ciba nomenclature) would be to the benefit of everybody. Androst-5-ene-3β-ol-17-one is not the only 17-ketosteroid with a double bond. The author does not always set his matter out clearly: in discussing the interpretation of the blood cholesterol level, the distinction between blood values and serum values becomes a little blurred and the calculations of the urea clearance seem to be needlessly complicated.

The book concludes with appendices containing useful information. In a hospital laboratory it might be more helpful to know the number of milligrams in a grain (which is not given) than the number of square metres in a square yard (which is). The ounce, which contains 28.35 grams, should be described as the ounce avoirdupois and the apothecaries ounce might well be included; I have yet to find a medical student who knows the difference between the weight of an ounce of sugar and of an ounce of strychnine.

In spite of these criticisms most hospital laboratories will wish to have a copy of this book because of its invaluable collection of methods.

ARTHUR JORDAN.

Textbook of Virology for Students and Practitioners of Medicine. 2nd ed. By A. J. Rhodes and C. E. van Rooyen. (Pp. xvi+562; 79 figures. 61s. 6d. London: Baillière, Tindall & Cox. 1953.

The first edition of this book, which contained 312 pages, appeared in 1949. The second edition now has 527 pages, a fair indication of the amount of new information which has been published, although it must be pointed out that owing to the delays of publication the latest references given are 1951. However, much of the material which the authors have included in a didactic fashion is of more recent vintage. Because so much of our knowledge is of recent origin, the authors have tried to cater for practising physicians, who probably had no instruction in virology while students, as well as for postgraduate and undergraduate students. The layout makes it easy to read, and the subdivision into diseases of the different anatomical systems has much to favour it. However, in the eyes of the reviewer, the authors and publisher, in their desire for completeness, have produced a book which is excessively expensive for students, particularly when our knowledge of the subject is altering so rapidly. The photographs of virus particles as seen in the electron microscope are very good,

but one doubts whether it is appropriate to take five pages of text to describe the workings of the electromicroscope, which only a small number of specialists can use. Similarly three pages of photographs of ultracentrifuges seem unnecessary.

I wonder how good the evidence was that "measles virus has been recovered from the brain" (p. 146) and there is even less reason to give physicians and students any reason to think rabbits and guinea-pigs are of value in the study of measles. I would also question the wisdom of making the unqualified statement that "measles virus can be grown on the chorio-allantois of the developing embryo or in tissue culture."

In spite of these few adverse comments, I should hasten to say that this is a useful adjunct to the growing number of books on viruses and rickettsiae. Sixty-five pages on poliomyelitis are a good indication of the increasing interest in this disease throughout the world, and this chapter is supported by extensive references. The detailed chapter on rabies should also be very useful to those faced with the problem of investigating suspected cases. There are numerous useful tables, and detailed instructions on such subjects as the control of poliomyelitis, and quarantine regulations for yellow fever and maps showing distribution of several diseases.

F. O. MacCallum.

Blood Groups in Man, 2nd ed. By R. R. Race and Ruth Sanger. (Pp. 400. 30s.) Oxford: Blackwell Scientific Publications. 1954.

When the first edition of this book was published it was immediately recognized as the standard work of reference on the genetical aspects of blood groups. Another four years' accumulation of knowledge has been built into this new edition, which is one-third as long again but costs no more.

It is astonishing that the authors manage to present such a plethora of blood group statistics without depressing the reader. The secret is that in almost all cases they succeed in fitting the facts into a reasonable scheme and manage to give the reader the feeling that blood groups follow orderly patterns.

In the few places where the authors present data that they have not themselves digested, the reader is immediately aware of the difference. Examples can be found on pages 198 to 202, where there is an attempt to reconcile the evidently irreconcilable data on the fractionation of Rh antisera; and on pages 34 to 36, where observations on the physico-chemical nature of the B-β combination are presented, which evidently baffle the authors as much as the ordinary reader.

There are a few conclusions which the authors seem to have arrived at on rather slender evidence, as when they say "blood survives a postal journey best in the form of a clot." For the rest one has the impression on page after page that the authors are writing on a subject which they know intimately, and one cannot help being delighted by the mastery of their subject which they constantly display. A particularly satisfying example occurs on page 251, where the amounts of Fy^a antigen within the genotype Fy^aFy^b are shown to fit an approximately normal distribution.

It is fascinating to learn on page 307 that, of 132 people working at the Lister Institute, 126 had combinations of blood groups which distinguished them from all their colleagues. "The day, foreseen long ago by Landsteiner, when blood would be known to be as individual as finger prints is now so close that it can be foreseen by ordinary people."

No laboratory seriously engaged in blood grouping, in whatever part of the world it is situated, can do without this book.

W. M. Mollison.

Pathology in Surgery. By Edwin F. Hirsch. (Pp. xvii +474; 388 photographs. 76s. 6d.) London: Baillière, Tindall & Cox. 1953.

Pathological atlases appear to be popular in the United States, as a similar atlas of surgical histology published by the same firm is advertised on the dust jacket of this book. So long as these atlases are used for reference only and not regarded as a short cut to histological diagnosis they can be quite valuable adjuncts, but no photographs or photomicrographs can replace the study of the actual specimen or section.

In this book the photographs are good and the photomicrographs are clear and representative. Occasionally the photomicrographs are repetitive; for example, there are four almost identical pictures of seminoma testis. Unfortunately the text does not reach the high standard of the illustrations. The language gives the impression of a poor translation and the arrangement of organs under systems is quite haphazard. Chapter 2 on the digestive system begins with a short summary of lesions of the lips and face and includes the tonsils, while the liver, gall bladder, bile ducts, and pancreas are dealt with quite separately in Chapter 7. Descriptions of both macroscopic and microscopic appearances are short and staccato and give the reader the feeling of being bombarded by so many facts that it is impossible to differentiate the important from the less important. Certain aspects of surgical pathology too are stressed which would not be considered in an English textbook. For example, in his general comments on bile ducts the author states:

"Perhaps the most fundamental element in the pathologist's fund of information covering the pathology of the common bile duct is his ability to recognize the duct as a tissue when he is confronted with a segment accidentally removed." Again in general comments on the liver there is a similar statement: "Unless a surgeon for some unusual reason, or by mistake, removes all or a large part of the liver, the tissues of the liver observed in surgical pathology are small biopsies."

It is not a book that can be recommended to students, but may be useful as a book of reference for surgical pathologists.

M. Gillespie.

Bacteriology for Students of Dental Surgery. By R. H. Lucas and I. R. H. Kramer. (Pp. viii+262; 56 figures. 22s. 6d.) London: J. & A. Churchill. 1954.

This textbook presents the problems of bacterial infection to the dental student in a simple and concise form. Systematic bacteriology has been limited mainly to general principles, and the morphology and cultural reactions of the bacteria are only briefly considered. Special attention is given to important dental problems, such as the bacteriology of dental caries and sterilization in dental practice.

This book should prove useful to the dental student. It is therefore hoped that, in future editions, the figure illustrating the tablet method for antibiotic sensitivity tests will be modified as, with a light inoculum, penicillin-resistant strains of *Staph. aureus* may give misleading results.

R. W. FAIRBROTHER.

Medical and Scientific Investigation in the Christie Case. By Francis E. Camps; with Foreword by the Attorney General, Sir Lionel Heald, Q.C., M.P. (Pp. xxiii+244; 47 illustrations, 6 coloured plates. 30s.) London: Medical Publications Ltd. 1953.

A series of murders is committed; the culprit is caught, tried, convicted, and hanged—and a book is born.

The production is worthy of its parentage, and, as the Attorney-General says in a short foreword, "The book is a tribute of sincere admiration to the skill, patience and team spirit of those responsible at all levels and in all departments for the brilliant work described." In fact, the book is a chronological detail of thoroughness which is nothing more than one expects. It is a tribute to the perfect liaison between police and pathologist.

The price is 30s.; is murder worth it?!

R. M. HEGGIE.

Arrest of Bleeding. By J. Roskam. (Pp. 63. 20s.) Oxford: Blackwell Scientific Publications.

This is a disappointing book. It is called *Arrest of Bleeding*, but is in fact a résumé of Professor Roskam's life work on the statistical study of the bleeding-time and its application in rabbits and to a lesser extent in man, and the appraisal of a number of alleged haemostatics. It is a good introduction to his work, but is often insufficient without reference to his original papers. Very rightly it emphasizes the great importance of the many factors in haemostasis other than the coagulation mechanism, but there is no wide critical survey of haemostasis. The translation is often irritating and occasionally incomprehensible. It is well produced but is expensive for only 63 pages of text.

J. R. O'BRIEN.

Two new scientific journals are announced, *The British Journal of Haematology* and *Clinical Chemistry*. *Clinical Chemistry* is to be published by Paul B. Hoeber Inc., 49, East 33rd Street, New York, 16, at an annual subscription of $9. *The British Journal of Haematology* will be edited by J. V. Dacie and published by Blackwell, of Oxford, at an annual subscription of £2 10s. The first number will appear in January.

Second International Congress of Neuropathology

The second international congress of neuropathology has been arranged for September 12–17, 1955, in London.

Offers of contributions should be sent to Dr. W. H. McMenemey, Maida Vale Hospital for Nervous Diseases, London, W.9, from whom further particulars may be obtained.

Correction

Professor Martin wishes to point out an error in the review article, "The Use and Limitations of Filter-paper Electrophoresis" (*J. clin. Path.*, 1954, **7**, 87). In Table I, page 95, the buffer formula for diethyl barbituric acid buffer should read:

1.84 g. diethylbarbituric acid
10.3 g. sodium diethyl barbiturate in 1 litre of water.

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